



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C07H 21/04, C12P 19/34	A1	(11) International Publication Number: WO 95/35390 (43) International Publication Date: 28 December 1995 (28.12.95)
(21) International Application Number: PCT/US95/07671 (22) International Filing Date: 14 June 1995 (14.06.95) (30) Priority Data: 08/263,937 22 June 1994 (22.06.94) US (60) Parent Application or Grant (63) Related by Continuation US 08/263,937 (CIP) Filed on 22 June 1994 (22.06.94) (71) Applicant (for all designated States except US): MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK [US/US]; One Gustave L. Levy Place, New York, NY 10029-6574 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): ZHANG, David, Y. [US/US]; 82-24 165th Street, Jamaica, NY 11432 (US). (74) Agent: SEIDE, Rochelle, K.; Brumbaugh, Graves, Donohue & Raymond, 30 Rockefeller Plaza, New York, NY 10112-0228 (US).		(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: LIGATION-DEPENDENT AMPLIFICATION FOR THE DETECTION OF INFECTIOUS PATHOGENS AND ABNORMAL GENES		
(57) Abstract An improved method allowing for rapid sensitive and standardized detection of a target nucleic acid from a pathogenic microorganism or virus or normal or abnormal gene in a sample is provided. The method involves hybridizing a target nucleic acid to several non-overlapping oligonucleotide probes that hybridize to adjacent regions in the target nucleic acid, the probes being referred to capture/amplification probes and amplification probes, respectively, in the presence of paramagnetic beads coated with a ligand binding moiety. Through the binding of a ligand attached to one end of the capture/amplification probe and the specific hybridization of portions of the probes to adjacent sequences in the target nucleic acid, a complex comprising the target nucleic acid, the probes and the paramagnetic beads is formed. The probes may then be ligated together to form a contiguous ligated amplification sequence bound to the beads, which, upon denaturation to remove the target nucleic acid and unligated probes, may be directly detected or amplified using a suitable amplification technique, e.g., PCR, for detection. The detection of the ligated amplification sequence, either directly or following amplification of the ligated amplification sequence, indicates the presence of the target nucleic acid in a sample.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

Description

Ligation-dependent Amplification For The Detection Of Infectious Pathogens And Abnormal Genes

The present application is a continuation-in-part of pending U.S. Application Serial No. 08/263,937 filed June 22, 1994.

5. Technical Field

The present invention relates to assays and kits for carrying out said assays for the rapid, automated detection of infectious pathogenic agents and normal and abnormal genes.

10

Background of the Invention

A number of techniques have been developed recently to meet the demands for rapid and accurate detection of infectious agents, such as viruses, bacteria and fungi, and detection of normal and abnormal genes. Such techniques, which generally involve the amplification and detection (and subsequent measurement) of minute amounts of target nucleic acids (either DNA or RNA) in a test sample, include inter alia the polymerase chain reaction (PCR) (Saiki, et al., Science 230:1350, 1985; Saiki et al., Science 239:487, 1988; PCR Technology, Henry A. Erlich, ed., Stockton Press, 1989; Patterson et al., Science 260:976, 1993), ligase chain reaction (LCR) (Barany, Proc. Natl. Acad. Sci. USA 88:189, 1991), strand displacement amplification (SDA) (Walker et al., Nucl. Acids Res. 20:1691, 1992), Q β replicase amplification (Q β RA) (Wu et al., Proc. Natl. Acad. Sci. USA 89:11769, 1992; Lomeli et al., Clin. Chem. 35:1826, 1989) and self-sustained replication (3SR) (Guatelli et al.,

Proc. Natl. Acad. Sci. USA 87:1874-1878, 1990). While all of these techniques are powerful tools for the detection and identification of minute amounts of a target nucleic acid in a sample, they all suffer from various problems, which have prevented their general applicability in the clinical laboratory setting for use in routine diagnostic techniques.

One of the most difficult problems is preparation of the target nucleic acid prior to carrying out its amplification and detection. This process is time and labor intensive and, thus, generally unsuitable for a clinical setting, where rapid and accurate results are required. Another problem, especially for PCR and SDA, is that conditions for amplifying the target nucleic acid for subsequent detection and optional quantitation vary with each test, i.e., there are no constant conditions favoring test standardization. This latter problem is especially critical for the quantitation of a target nucleic acid by competitive PCR and for the simultaneous detection of multiple target nucleic acids.

Circumvention of the aforementioned problems would allow for development of rapid standardized assays, utilizing the various techniques mentioned above, that would be particularly useful in performing epidemiologic investigations, as well as in the clinical laboratory setting for detecting pathogenic microorganisms and viruses in a patient sample. Such microorganisms cause infectious diseases that represent a major threat to human health. The development of standardized and automated analytical techniques and kits therefor, based on rapid and sensitive identification of target nucleic acids specific for an infectious disease agent would provide advantages over techniques involving immunologic or culture detection of bacteria and viruses.

Reagents may be designed to be specific for a particular organism or for a range of related organisms. These reagents could be utilized to directly assay microbial genes conferring resistance to various antibiotics and virulence factors resulting in disease. Development of rapid standardized analytical techniques will aid in the selection of the proper treatment.

In some cases, assays having a moderate degree of sensitivity (but high specificity) may suffice, e.g., in initial screening tests. In other cases, great sensitivity (as well as specificity) is required, e.g., the detection of the HIV genome in infected blood may require finding the virus nucleic acid sequences present in a sample of one part per 10 to 100,000 human genome equivalents (Harper et al., Proc. Nat'l. Acad. Sci., USA 83:772, 1986).

Blood contaminants, including inter alia, HIV, HTLV-I, hepatitis B and hepatitis C, represent a serious threat to transfusion patients and the development of routine diagnostic tests involving the nucleic acids of these agents for the rapid and sensitive detection of such agents would be of great benefit in the clinical diagnostic agree laboratory. For example, the HIV genome can be detected in a blood sample using PCR techniques, either as an RNA molecule representing the free viral particle or as a DNA molecule representing the integrated provirus (Ou et al., Science 239:295, 1988; Murakawa et al., DNA 7:287, 1988).

In addition, epidemiologic investigations using classical culturing techniques have indicated that disseminated Mycobacterium avium-intracellulaire (MAI) infection is a complication of late-stage Acquired Immunodeficiency Syndrome (AIDS) in children and adults. The precise extent of the problem is not

clear, however, since current cultural methods for detecting mycobacteria are cumbersome, slow and of questionable sensitivity. Thus, it would be desirable and highly beneficial to devise a rapid, sensitive and specific technique for MAI detection in order to provide a definitive picture of the involvement in HIV-infected and other immunosuppressed individuals. Such studies must involve molecular biological methodologies, based on detection of a target nucleic acid, which have routinely been shown to be more sensitive than standard culture systems (Boddinghaus et al., J. Clin. Med. 28:1751, 1990).

Other applications for such techniques include detection and characterization of single gene genetic disorders in individuals and in populations (see, e.g., Landergren et al., Science 241: 1077, 1988 which discloses a ligation technique for detecting single gene defects, including point mutations). Such techniques should be capable of clearly distinguishing single nucleotide differences (point mutations) that can result in disease (e.g., sickle cell anemia) as well as deleted or duplicated genetic sequences (e.g., thalassemia).

The methods referred to above are relatively complex procedures that, as noted, suffer from drawbacks making them difficult to use in the clinical diagnostic laboratory for routine diagnosis and epidemiological studies of infectious diseases and genetic abnormalities. All of the methods described involve amplification of the target nucleic acid to be detected. The extensive time and labor required for target nucleic acid preparation, as well as variability in amplification templates (e.g., the specific target nucleic acid whose detection is being measured) and conditions, render such procedures unsuitable for

standardization and automation required in a clinical laboratory setting.

The present invention is directed to the development of rapid, sensitive assays useful for the detection and monitoring of pathogenic organisms, as well as the detection of abnormal genes in an individual. Moreover, the methodology of the present invention can be readily standardized and automated for use in the clinical laboratory setting.

Summary Of The Invention

An improved method, which allows for rapid, sensitive and standardized detection and quantitation of nucleic acids from pathogenic microorganisms from samples from patients with infectious diseases has now been developed. The improved methodology also allows for rapid and sensitive detection and quantitation of genetic variations in nucleic acids in samples from patients with genetic diseases or neoplasia.

This method provides several advantages over prior art methods. The method simplifies the target nucleic acid isolation procedure, which can be performed in microtubes, microchips or micro-well plates, if desired. The method allows for isolation, amplification and detection of nucleic acid sequences corresponding to the target nucleic acid of interest to be carried out in the same sample receptacle, e.g., tube or micro-well plate. The method allows for standardization of conditions, because, only a pair of generic amplification probes may be utilized in the present method for detecting a variety of target nucleic acids. The amplification probes, which in the method may be covalently joined end to end, form a contiguous ligated amplification sequence. This ligated amplification sequence, rather than the target nucleic acid, is either directly detected or amplified,

allowing for substantially the same amplification conditions to be used for a variety of different infectious agents and, thus, leading to more controlled and consistent results being obtained. In addition,
5 multiple infectious agents in a single sample may be detected using the methodology disclosed.

Additional advantages of the present invention include the ability to automate the protocol of the method disclosed, which is important in performing
10 routine assays, especially in the clinical laboratory and the ability of the method to utilize various nucleic acid amplification systems, e.g., polymerase chain reaction (PCR), strand displacement amplification (SDA), ligase chain reaction (LCR), self-sustained
15 sequence replication (3SR) and Q β replicase amplification (Q β RA).

The present method incorporates magnetic separation techniques using paramagnetic particles or beads coated with a ligand binding moiety that
20 recognizes and binds to a ligand on an oligonucleotide capture probe to isolate a target nucleic acid (DNA or RNA) from a sample of a clinical specimen containing e.g., a suspected pathogenic microorganism or gene abnormality, in order to facilitate detection of the
25 underlying disease-causing agent.

In one aspect of the present invention, a target nucleic acid is hybridized to a pair of non-overlapping oligonucleotide amplification probes in the presence of paramagnetic beads coated with a ligand binding moiety,
30 e.g., streptavidin, to form a complex. These probes are referred to as a capture/amplification probe and an amplification probe, respectively. The capture/amplification probe contains a ligand, e.g., biotin, that is recognized by and binds to the ligand
35 binding moiety on the paramagnetic beads. The probes are designed so that each contains generic sequences

(i.e., not target nucleic acid specific) and specific sequences complementary to a nucleotide sequence in the target nucleic acid. The specific sequences of the probes are complementary to adjacent regions of the target nucleic acid, and thus do not overlap one another. Subsequently, the two probes may be joined together using a ligating agent to form a contiguous ligated amplification sequence. The ligating agent may be an enzyme, e.g., DNA ligase or a chemical. Following washing and removal of unbound reactants and other materials in the sample, the detection of the target nucleic acid in the original sample is determined by detection of the ligated amplification sequence. The ligated amplification sequence may be directly detected if a sufficient amount (e.g., 10^6 - 10^7 molecules) of target nucleic acid was present in the original sample. If an insufficient amount of target nucleic acid ($< 10^6$ molecule) was present in the sample, the ligated amplification sequence (not the target nucleic acid) may be amplified using suitable amplification techniques, e.g. PCR, for detection. Alternatively, capture and amplification functions may be performed by separate and independent probes. Unligated probes, as well as the target nucleic acid, are not amplified in this technique.

Brief Description Of The Drawings

Fig. 1 is a generic schematic diagram showing the various components used in the present method of capture, ligation-dependent amplification and detection of a target nucleic acid.

Fig. 2 is a schematic flow diagram generally showing the various steps in the present method.

Fig. 3 is an autoradiograph depicting the detection of a PCR amplified probe that detects HIV-1

RNA. Lane A is the ligated amplification sequence according to the invention; Lane B, which is a control, is PCR amplified nanovariant DNA, that does not contain any HIV-1-specific sequences.

5 Fig. 4 is a schematic diagram of an embodiment of the present invention showing the various components used for capture and ligation-dependent detection of a target nucleic acid, e.g. HCV RNA, and subsequent
10 amplification of its sequences, employing two capture/amplification probes containing a bound biotin moiety and two ligation-dependent amplification probes.

 Fig. 5 is a schematic flow diagram showing magnetic isolation, target specific ligation and PCR
15 amplification for the detection of HCV RNA using a single capture/amplification probe and two amplification probes.

 Fig. 6 is a schematic diagram showing the various components used to amplify and detect a target nucleic acid e.g. HCV RNA, employing two capture/amplification
20 probes, each containing a bound biotin moiety, and a single amplification probe.

 Fig. 7 is a schematic diagram showing various components used to detect a target nucleic acid e.g. HCV RNA, employing two capture/amplification probes,
25 each containing a bound biotin moiety, and a single amplification probe that circularizes upon hybridization to the target nucleic acid and ligation of free termini.

 Fig. 8 is a photograph of ethidium bromide stained
30 DNA depicting PCR amplified probes used to detect HCV RNA in a sample. The amount of HCV RNA in the sample is determined by comparing sample band densities to those of standard serial dilutions of HCV transcripts.

 Fig. 9. is a photograph of ethidium bromide
35 stained DNA depicting PCR amplified single, full length ligation-dependent and circularizable probes used to

detect HCV RNA in a sample. The amount of HCV RNA in the sample is determined by comparing sample band densities to those of standard serial dilutions of HCV transcripts.

5

Detailed Description Of The Invention

The present invention is directed towards simplified sample preparation and generic amplification systems for use in clinical assays to detect and
10 monitor pathogenic microorganisms in a test sample, as well as to detect abnormal genes in an individual. Generic amplification systems are described for clinical use that combine magnetic separation techniques with ligation/amplification techniques for
15 detecting and measuring nucleic acids in a sample. The separation techniques may be combined with most amplification systems, including inter alia, PCR, LCR, SDA and Q β amplification techniques. The advantages of the present invention include (1) suitability for
20 clinical laboratory settings, (2) ability to obtain controlled and consistent (standardizable) results, (3) ability to quantitate nucleic acids in a particular sample and (4) ability to simultaneously detect and quantitate multiple target nucleic acids in a test
25 sample. Moreover, the complete protocol of the presently disclosed method may be easily automated, making it useful for routine diagnostic testing in a clinical laboratory setting.

The present invention incorporates magnetic
30 separation, utilizing paramagnetic particles, beads or spheres that have been coated with a ligand binding moiety that recognizes and binds to ligand present on an oligonucleotide capture probe, described below, to isolate a target nucleic acid (DNA or RNA) from a
35 clinical sample in order to facilitate its detection.

Magnetic separation is a system that uses paramagnetic particles or beads coated with a ligand binding moiety to isolate a target nucleic acid (RNA or DNA) (Lomeli *et al.* Clin. Chem. 35:1826, 1989) from a sample. The principle underscoring this method is one of hybrid formation between a capture probe containing a ligand, and a target nucleic acid through the specific complementary sequence between the probe and target. Hybridization is carried out in the presence of a suitable chaotropic agent, e.g., guanidine thiocyanate (GnSCN) which facilitates the specific binding of the probe to complementary sequences in the target nucleic acid. The hybrid so formed is then captured on the paramagnetic bead through specific binding of the ligand on the capture probe to the ligand binding moiety on the bead.

The term "ligand" as used herein refers to any component that has an affinity for another component termed here as "ligand binding moiety." The binding of the ligand to the ligand binding moiety forms an affinity pair between the two components. For example, such affinity pairs include, inter alia, biotin with avidin/streptavidin, antigens or haptens with antibodies, heavy metal derivatives with thiogroups, various polynucleotides such as homopolynucleotides as poly dG with poly dC, poly dA with poly dT and poly dA with poly U. Any component pairs with strong affinity for each other can be used as the affinity pair, ligand-ligand binding moiety. Suitable affinity pairs are also found among ligands and conjugates used in immunological methods. The preferred ligand-ligand binding moiety for use in the present invention is the biotin/streptavidin affinity pair.

In one aspect, the present invention provides for the capture and detection of a target nucleic acid as depicted in Fig. 1, which provides a schematic

depiction of the capture and detection of a target nucleic acid. In the presence of paramagnetic beads or particles (a) coated with a ligand binding moiety (b), the target nucleic acid is hybridized simultaneously to a pair of oligonucleotide amplification probes, i.e., a first nucleotide probe (also referred to as a capture/amplification probe) and a second nucleotide probe (also referred to as an amplification probe), designated in Fig. 1 as Capture/Amp-probe-1 (d and e) and Amp-probe-2 (f and g), respectively. The probes may be either oligodeoxyribonucleotide or oligoribonucleotide molecules, with the choice of molecule type depending on the subsequent amplification method, e.g., PCR (deoxyribonucleotides) or Q β replicase (either deoxyribo- or ribonucleotides) to be used.

The capture/amplification probe is designed to have a generic 3' nucleotide sequence (d), i.e., it is not specific for the specific target nucleic acid being analyzed and thus can be used with a variety of target nucleic acids. In other words, the 3' sequence of the first probe is not complementary, nor hybridizable, to the nucleotide sequence of the target nucleic acid. The 5' portion (e) of the capture/amplification probe comprises a nucleotide sequence that is complementary and hybridizable to a portion of the nucleotide sequence of the specific target nucleic acid. Preferably, for use with pathogenic microorganisms and viruses, the capture/amplification probe is synthesized so that its 3' generic sequence (d) is the same for all systems, with the 5' specific sequence (e) being specifically complementary to a target nucleic acid of an individual species or subspecies of organism or an abnormal gene, e.g. the gene(s) responsible for cystic fibrosis or sickle cell anemia. In certain instances, it may be desirable that the 5' specific portion of the

capture/amplification probe be specifically complementary to the nucleotide sequence of a target nucleic acid of a particular strain of organism.

Capture/Amp-probe-1 further contains a ligand (c) at the 3' end of the probe (d), which is recognized by and binds to the ligand binding moiety (b) coated onto the paramagnetic beads (a).

The second or amplification probe, i.e., Amp-probe-2 in Fig. 1, contains a 3' sequence (f) that is complementary and hybridizes to a portion of the nucleotide sequence of a target nucleic acid immediately adjacent to (but not overlapping) the sequence of the target that hybridizes to the 5' end of Capture/Amp-probe-1. Amp-probe-2 also contains a 5' generic sequence (g) which is neither complementary nor hybridizable to the target nucleic acid, to which may be optionally attached at the 5' end thereof a label or signal generating moiety (***). Such signal generating moieties include, inter alia, radioisotopes, e.g., ^{32}P or ^3H , fluorescent molecules, e.g., fluorescein and chromogenic molecules or enzymes, e.g., peroxidase. Such labels are used for direct detection of the target nucleic acid and detects the presence of Amp-probe-2 bound to the target nucleic acid during the detection step. ^{32}P is preferred for detection analysis by radioisotope counting or autoradiography of electrophoretic gels. Chromogenic agents are preferred for detection analysis, e.g., by an enzyme linked chromogenic assay.

As a result of the affinity of the ligand binding moiety on the paramagnetic beads for the ligand on the capture/amplification probe, target nucleic acid hybridized to the specific 5' portion of the probe is captured by the paramagnetic beads. In addition, Amp-probe-2, which has also hybridized to the target

nucleic acid is also captured by the paramagnetic beads.

After capture of the target nucleic acid and the two hybridized probes on the paramagnetic beads, the probes are ligated together (at the site depicted by the verticle arrow in Fig. 1) using a ligating agent to form a contiguous single-stranded oligonucleotide molecule, referred to herein as a ligated amplification sequence. The ligating agent may be an enzyme, e.g., a DNA or RNA ligase, or a chemical joining agent, e.g., cyanogen bromide or a carbodiimide (Sokolova et al., FEBS Lett. 232:153-155, 1988). The ligated amplification sequence is hybridized to the target nucleic acid (either an RNA or DNA) at the region of the ligated amplification sequence that is complementary to the target nucleic acid (e.g., (e) and (f) in Fig. 1).

If a sufficient amount of target nucleic acid (e.g., 10^6 - 10^7 molecules) is present in the sample, detection of the target nucleic acid can be achieved without any further amplification of the ligated amplification sequence, e.g., by detecting the presence of the optional signal generating moiety of at the 5' end of Amp-probe-2.

If there is insufficient target nucleic acid (e.g., $<10^6$ molecules) in the sample for direct detection, as above, the ligated amplification sequence formed as described above by the ligation of Capture/Amp-probe-1 and Amp-probe-2 may be amplified for detection as described below.

The present method may be used with routine clinical samples obtained for testing purposes by a clinical diagnostic laboratory. Clinical samples that may be used in the present method include, inter alia, whole blood, separated white blood cells, sputum, urine, tissue biopsies, throat swabbings and the like,

i.e., any patient sample normally sent to a clinical laboratory for analysis.

With reference to Fig. 2, which provides a general diagrammatic description of the magnetic separation and target-dependent detection of a target nucleic acid in a sample, this aspect of the present method involves the following steps:

(a) The first step is the capture or isolation of a target nucleic acid present in the sample being analyzed, e.g., serum. A suitable sample size for analysis that lends itself well to being performed in a micro-well plate is about 100 μ l. The use of micro-well plates for analysis of samples by the present method facilitates automation of the method. The sample, containing a suspected pathogenic microorganism or virus or abnormal gene, is incubated with an equal volume of lysis buffer, containing a chaotropic agent (i.e., an agent that disrupts hydrogen bonds in a compound), a stabilizer and a detergent, which provides for the release of any nucleic acids and proteins that are present in the sample. For example, a suitable lysis buffer for use in the present method comprises 2.5 - 5M guanidine thiocyanate (GnSCN), 10% dextran sulfate, 100mM EDTA, 200mM Tris-HCl(pH 8.0) and 0.5% NP-40 (Nonidet P-40, a nonionic detergent, N-lauroylsarcosine, Sigma Chemical Co., St. Louis, MO). The concentration of GnSCN, which is a chaotropic agent, in the buffer also has the effect of denaturing proteins and other molecules involved in pathogenicity of the microorganism or virus. This aids in preventing the possibility of any accidental infection that may occur during subsequent manipulations of samples containing pathogens.

Paramagnetic particles or beads coated with the ligand binding moiety are added to the sample, either simultaneous with or prior to treatment with the lysis

buffer. The paramagnetic beads or particles used in the present method comprise ferricoxide particles (generally < 1 um in diameter) that possess highly convoluted surfaces coated with silicon hydrides. The
5 ligand binding moiety is covalently linked to the silicon hydrides. The paramagnetic particles or beads are not magnetic themselves and do not aggregate together. However, when placed in a magnetic field, they are attracted to the magnetic source.
10 Accordingly, the paramagnetic particles or beads, together with anything bound to them, may be separated from other components of a mixture by placing the reaction vessel in the presence of a strong magnetic field provided by a magnetic separation device. Such
15 devices are commercially available, e.g., from Promega Corporation or Stratagene, Inc.

Suitable paramagnetic beads for use in the present method are those coated with streptavidin, which binds to biotin. Such beads are commercially available from
20 several sources, e.g., Streptavidin MagneSphere® paramagnetic particles obtainable from Promega Corporation and Streptavidin-Magnetic Beads (catalog #MB002) obtainable from American Qualex, La Mirada, CA.

Subsequently, a pair of oligonucleotide
25 amplification probes, as described above, is added to the lysed sample and paramagnetic beads. In a variation, the probes and paramagnetic beads may be added at the same time. As described above, the two oligonucleotide probes are a first probe or
30 capture/amplification probe (designated Capture/Amp-probe-1 in Fig. 1) containing a ligand at its 3' end and a second probe or amplification probe (designated Amp-probe-2 in Fig. 1). For use with streptavidin-coated paramagnetic beads, the first probe is
35 preferably a 3'-biotinylated capture/amplification probe.

The probes may be synthesized from nucleoside triphosphates by known automated oligonucleotide synthetic techniques, e.g., via standard phosphoramidite technology utilizing a nucleic acid synthesizer. Such synthesizers are available, e.g., from Applied Biosystems, Inc. (Foster City, CA).

Each of the oligonucleotide probes are about 40-200 nucleotides in length, preferably about 50-100 nucleotides in length, which, after ligation of the probes, provides a ligated amplification sequence of about 80-400, preferably 100-200, nucleotides in length, which is suitable for amplification via PCR, Q β replicase or SDA reactions.

The target nucleic acid specific portions of the probes, i.e., the 5' end of the first capture/amplification probe and the 3' end of the second amplification probe complementary to the nucleotide sequence of the target nucleic acid, are each approximately 15-60 nucleotides in length, preferably about 18-35 nucleotides, which provides a sufficient length for adequate hybridization of the probes to the target nucleic acid.

With regard to the generic portions of the probes, i.e., the 3' end of the capture/amplification probe and the 5' end of the amplification probe, which are not complementary to the target nucleic acid, the following considerations, inter alia, apply:

(1) The generic nucleotide sequence of an oligodeoxynucleotide capture/amplification probe comprises at least one and, preferably two to four, restriction endonuclease recognition sequences(s) of about six nucleotides in length, which can be utilized, if desired, to cleave the ligated amplification sequence from the paramagnetic beads by specific restriction endonucleases, as discussed below.

Preferred restriction sites include, inter alia, *EcoRI* (GAATTC), *SmaI* (CCCGGG) and *HindIII* (AAGCTT).

(2) The generic nucleotide sequence comprises a G-C rich region which, upon hybridization to a primer, as discussed below, provides a more stable duplex molecule, e.g., one which requires a higher temperature to denature. Ligated amplification sequences having G-C rich generic portions of the capture/amplification and amplification probes may be amplified using a two temperature PCR reaction, wherein primer hybridization and extension may both be carried out at a temperature of about 60-65°C (as opposed to hybridizing at 37°C, normally used for PCR amplification) and denaturation at a temperature of about 92°C, as discussed below.

The use of a two temperature reaction reduces the length of each PCR amplification cycle and results in a shorter assay time.

Following incubation of the probes, magnetic beads and target nucleic acid in the lysis buffer for about 30-60 minutes, at a temperature of about 37°C, a ternary complex comprising the target nucleic acid and hybridized probes is formed, which is bound to the paramagnetic beads through the binding of the ligand (e.g., biotin) on the capture/amplification probe to the ligand binding moiety (e.g., streptavidin) on the paramagnetic beads. The method is carried out as follows:

(a) The complex comprising target nucleic acid-probes-beads is then separated from the lysis buffer by means of a magnetic field generated by a magnetic device, which attracts the beads. The magnetic field is used to hold the complex to the walls of the reaction vessel, e.g., a micro-well plate or a microtube, thereby allowing for the lysis buffer and any unbound reactants to be removed, e.g., by decanting, without any appreciable loss of target

nucleic acid or hybridized probes. The complex is then washed 2-3 times in the presence of the magnetic field with a buffer that contains a chaotropic agent and detergent in amounts that will not dissociate the complex. A suitable washing buffer for use in the present method comprises about 1.0 - 1.5M GnSCN, 10mM EDTA, 100mM Tris-HCl (pH 8.0) and 0.5% NP-40 (Nonidet P-40, nonionic detergent, Sigma Chemical Co., St. Louis, MO). Other nonionic detergents, e.g., Triton X-100, may also be used. The buffer wash removes unbound proteins, nucleic acids and probes that may interfere with subsequent steps. The washed complex may be then washed with a solution of KCl to remove the GnSCN and detergent and to preserve the complex. A suitable concentration of KCl is about 100 to 500mM KCl. Alternatively, the KCl wash step may be omitted in favor of two washes with ligase buffer.

(b) If the probes are to be ligated together, the next step in the present method involves treating the complex from step (a) with a ligating agent that will join the two probes. The ligating agent may be an enzyme, e.g., DNA or RNA ligase, or a chemical agent, e.g., cyanogen bromide or a carbodiimide. This serves to join the 5' end of the first oligonucleotide probe to the 3' end of the second oligonucleotide probe (capture/amplification probe and amplification probe, respectively) to form a contiguous functional single-stranded oligonucleotide molecule, referred to herein as a ligated amplification sequence. The presence of the ligated amplification sequence detected, (via the signal generating moiety at the 5'-end of Amp-probe-2), indirectly indicates the presence of target nucleic acid in the sample. Alternatively, the ligated amplification sequence serves as the template for any of various amplification systems, such as PCR, SDA or Q β replicase amplification. Any of the

first and second probes which remain unligated after treatment are not amplified in subsequent steps in the method. Capture/amplification and amplification oligodeoxynucleotide probes may be ligated using a suitable ligating agent, such as a DNA or RNA ligase. Alternatively, the ligating agent may be a chemical, such as cyanogen bromide or a carbodiimide (Sokolova et al., FEBS Lett. 232:153-155, 1988). Preferred DNA ligases include T₄ DNA ligase and the thermostable Taq DNA ligase, with the latter being most preferable, for probes being subjected to amplification using PCR techniques. The advantage of using the Taq DNA ligase is that it is active at elevated temperatures (65-72°C). Joining the oligonucleotide probes at such elevated temperatures decreases non-specific ligation. Preferably, the ligation step is carried out for 30-60 minutes at an elevated temperature (about 65-72°C), after which time any unligated second amplification probe (Amp-probe-2 in Fig. 1) may be, optionally, removed under denaturing conditions.

Denaturation is performed after the ligation step by adding TE Buffer (10mM Tris-HCl pH 7.5, 0.1mM EDTA) to the mixture. The temperature of the mixture is then raised to about 92-95°C for about 1-5 minutes to denature the hybridized nucleic acid. This treatment separates the target nucleic acid (and unligated Amp-probe-2) from the hybridized ligated amplification sequences, which remains bound to the paramagnetic beads. In the presence of a magnetic field, as above, the bound ligated amplification sequence is washed with TE Buffer at elevated temperature to remove denatured target nucleic acid and any unligated Amp-probe-2 and resuspended in TE Buffer for further analysis.

(c) The third step in the process is detection of the ligated amplification sequence, which indicates the presence of the target nucleic acid in

the original test sample. This may be performed directly if sufficient target nucleic acid (about 10^6 - 10^7 molecules) is present in the sample or following amplification of the ligated amplification sequence, using one of the various amplification techniques, e.g., PCR, SDA, or Q β replicase amplification. For example, direct detection may be used to detect HIV-1 RNA in a serum sample from an acutely infected AIDS patient. Such a serum sample is believed to contain about 10^6 copies of the viral RNA/ml.

For direct detection, an oligonucleotide detection probe of approximately 10-15 nucleotides in length, prepared by automative synthesis as described above to be complementary to the 5' end of the Amp-probe-2 portion of the ligated amplification sequence, may be added to the ligated amplification sequence attached to the paramagnetic beads. The detection probe, which is labelled with a signal generating moiety, e.g., a radioisotope, a chromogenic agent or a fluorescent agent, is incubated with the complex for a period of time and under conditions sufficient to allow the detection probe to hybridize to the ligated amplification sequence. The incubation time can range from about 1-60 minutes and may be carried out at a temperature of about 4-60°C. Preferably, when the label is a fluorogenic agent, the incubation temperature is about 4°C; a chromogenic agent, about 37°C; and a radioisotope, about 37° - 60°C. Preferred signal generating moieties include, inter alia, ^{32}P (radioisotope), peroxidase (chromogenic) and fluorescein, acridine or ethidium (fluorescent).

Alternatively, for direct detection, as discussed above, the Amp-probe-2 itself may be optionally labeled at its 5' end with a signal generating moiety, e.g., ^{32}P . The signal generating moiety will then be incorporated into the ligated amplification sequence

following ligation of the Capture/Amp-probe-1 and Amp-probe-2. Thus, direct detection of the ligated amplification sequence, to indicate the presence of the target nucleic acid, can be carried out immediately following ligation and washing.

Any suitable technique for detecting the signal generating moiety directly on the ligated amplification probe or hybridized thereto via the detection primer may be utilized. Such techniques include scintillation counting (for ^{32}P) and chromogenic or fluorogenic detection methods as known in the art. For example, suitable detection methods may be found, *inter alia*, in Sambrook *et al.*, Molecular Cloning - A Laboratory Manual, 2d Edit., Cold Spring Harbor Laboratory, 1989, in Methods in Enzymology, Volume 152, Academic Press (1987) or Wu *et al.*, Recombinant DNA Methodology, Academic Press (1989).

If an insufficient amount of target nucleic acid is present in the original sample ($<10^6$ molecules), an amplification system is used to amplify the ligated amplification sequence for detection.

For example, the Q β replicase amplification technique may be used to amplify either oligoribonucleotide or oligodeoxyribonucleotide ligated amplification sequences using the protocol generally described in Zhang, D.Y., Q β Replicase Directed RNA Polymerization, Ph.D. Thesis Dissertation, New York University, 1992. Using the Q β technique, following ligation, the enzyme Q β replicase is added directly to the reaction vessel to amplify the ligated amplification sequence (*i.e.*, the ligated Capture/Amp-probe-1 and Amp-probe-2 in Fig. 1).

Briefly, Q β replicase amplification may be generally carried out as follows:

Between 10^2 and 10^9 molecules (*e.g.*, about 0.5 attograms to 0.5 nanograms) of ligated amplification

sequence are incubated with 48-50 $\mu\text{g/ml}$ Q β replicase (about 7×10^{10} active enzyme molecules) for 20 to 60 min at 37°C in 10 to 25 μl of 400 μM ATP, 400 μM CTP or 40 μM [α - ^{32}P]CTP, 400 μM GTP, 5 to 12mM MgCl_2 , and 90mM Tris-HCl (pH 7.5). The reactions are terminated by adding 10 to 80 μl of a stop buffer (20mM EDTA (pH7.5), 120mM NaCl) and placing them in an ice bath. Small aliquots are then removed to determine the yield and homogeneity of the oligoribonucleotide product.

For kinetic analysis, about 12.5 to 25 μl reactions are initiated with about 10^2 to 10^8 molecules of ligated amplification sequence. 2 to 4 μl samples are taken at various times and diluted with 18 to 30 μl of stop buffer. 8 μl of diluted sample are precipitated by the addition of 50 μl of an ice-cold precipitation solution (300mM phosphoric acid, 20mM sodium pyrophosphate, and 1mM EDTA). The precipitated RNA samples are bound to a nitrocellulose membrane (Bio-Rad Corp., Richmond, CA) on a dot-blot vacuum filtering manifold (Bio-Rad Corp.) and each is washed three times with 150 μl of ice-cold precipitation solution to remove unincorporated [α - ^{32}P]CTP. The membrane is then soaked in 150 ml of ice-cold precipitation solution for 10 min. The amount of [^{32}P] RNA present in each sample is determined by measuring the radioactivity of each dot-blot. The size of the RNA products in selected samples is determined by analyzing the remainder of the terminated reaction mixture by electrophoresis through an 8% polyacrylamide gel.

Alternatively, if the probes used in the present method are oligodeoxyribonucleotide molecules, PCR methodology can be employed to amplify the ligated amplification sequence, using known techniques (see, e.g., PCR Technology, H.A. Erlich, ed., Stockton Press, 1989, Sambrook et al., Molecular Cloning - A Laboratory

Manual, 2d Edit., Cold Spring Harbor Laboratory, 1989. When using PCR for amplification, two primers are employed, the first of the primers being complementary to the generic 3' end of Capture/Amp-probe-1 region of the ligated amplification sequence and the second primer corresponding in sequence to the generic 5' end of Amp-probe-2 portion of the ligated amplification sequence. These primers, like the sequences of the probes to which they bind, are designed to be generic and may be used in all assays, irrespective of the sequence of the target nucleic acid. Because the first primer is designed to anneal to the generic sequence at the 3' end of the ligated amplification sequence and the second primer corresponds in sequence to the generic sequence at the 5' end of the ligated amplification sequence, generic primers may be utilized to amplify any ligated amplification sequence.

A generic pair of PCR oligonucleotide primers for use in the present method may be synthesized from nucleoside triphosphates by known automated synthetic techniques, as discussed above for synthesis of the oligonucleotide probes. The primers may be 10-60 nucleotides in length. Preferably the oligonucleotide primers are about 18-35 nucleotides in length, with lengths of 16-21 nucleotides being most preferred. The pair of primers are designated to be complementary to the generic portions of the first capture/amplification probe and second amplification probe, respectively and thus have high G-C content. It is also preferred that the primers are designed so that they do not have any secondary structure, i.e., each primer contains no complementary region within itself that could lead to self annealing.

The high G-C content of the generic PCR primers and the generic portions of the ligated amplification sequence permits performing the PCR reaction at two

temperatures, rather than the usual three temperature method. Generally, in the three temperature method, each cycle of amplification is carried out as follows:

5 Annealing of the primers to the ligated amplification sequence is carried out at about 37-50°C; extension of the primer sequence by Taq polymerase in the presence of nucleoside triphosphates is carried out at about 70-75°C; and the denaturing step to release the extended primer is carried out at about 90-95°C. In
10 the two temperature PCR technique, the annealing and extension steps may both be carried at about 60-65°C, thus reducing the length of each amplification cycle and resulting in a shorter assay time.

For example, a suitable three temperature PCR
15 amplification (as provided in Saiki et al., Science 239:487-491, 1988) may be carried out as follows:

Polymerase chain reactions (PCR) are carried out in about 25-50 μ l samples containing 0.01 to 1.0 ng of template ligated amplification sequence, 10 to 100 pmol
20 of each generic primer, 1.5 units of Taq DNA polymerase (Promega Corp.), 0.2mM dATP, 0.2mM dCTP, 0.2mM dGTP, 0.2mM dTTP, 15mM MgCl₂, 10mM Tris-HCl (pH 9.0), 50mM KCl, 1 μ g/ml gelatin, and 10 μ l/ml Triton X-100 (Saiki, 1988). Reactions are incubated at 94°C for 1 minute,
25 about 37 to 55°C for 2 minutes (depending on the identity of the primers), and about 72°C for 3 minutes and repeated for 30-40, preferably 35, cycles. A 4 μ l-aliquot of each reaction is analyzed by electrophoresis through a 2% agarose gel and the DNA products in the
30 sample are visualized by staining the gel with ethidium-bromide.

The two temperature PCR technique, as discussed above, differs from the above only in carrying out the annealing/extension steps at a single temperature,
35 e.g., about 60-65°C for about 5 minutes, rather than at two temperatures.

Also, with reference to Fig. 2, quantitative detection of the target nucleic acid using a competitive PCR assay may also be carried out. For such quantitative detection, a oligodeoxyribonucleotide releasing primer, synthesized generally as described above, is added to the paramagnetic bead-bound ligated amplification sequence. The releasing primer, may or may not be but, preferably, will be the same as the first PCR primer discussed above. The releasing primer is designed to hybridize to the generic 3' end of the Capture/Amp-probe-1 portion of the ligated amplification sequence, which, as discussed above, comprises a nucleotide sequence recognized by at least one, and preferably two-four, restriction endonucleases to form at least one, and preferably two-four, double-stranded restriction enzyme cleavage site, e.g., a *EcoRI*, *SmaI* and/or *HindIII* site(s).

In this regard, as noted above, for use in a quantitative PCR amplification and detection system, it is important that the Capture/Amp-probe-1 be synthesized with at least one, and preferably two to four nucleotide sequences recognized by a restriction enzyme located at the 3' end of the probe. This provides the nucleotide sequences to which the releasing primer binds to form double-stranded restriction enzyme cleavage site(s).

After ligating the first and second probes to form the ligated amplification sequence, the releasing primer is hybridized to the ligated amplification sequence. At least one restriction enzyme, e.g., *EcoRI*, *SmaI* and/or *HindIII*, is then added to the hybridized primer and ligated amplification sequence. The ligated amplification sequence is released from the beads by cleavage at the restriction enzyme, e.g., *EcoRI* site.

Following its release from the beads, the ligated amplification sequence is serially diluted and then quantitatively amplified via the DNA Taq polymerase using a suitable PCR amplification technique, as
5 described above.

Quantitation of the original target nucleic acid in the sample may be performed by a competitive PCR method to quantitatively amplify the ligated amplification sequence, as provided, e.g., in Sambrook
10 et al., Molecular Cloning - A Laboratory Manual, 2d Edit., Cold Spring Harbor Laboratory, 1989.

In general, the method involves coamplification of two templates: the ligated amplification sequence and a control (e.g., the generic portions of the ligated
15 amplification sequence or the generic portions that have interposed thereto a nucleotide sequence unrelated to the sequence of the target nucleic acid) added in known amounts to a series of amplification reactions. While the control and ligated amplification sequence
20 are amplified by the same pair of generic PCR primers, the control template is distinguishable from the ligated amplification sequence, e.g., by being different in size. Because the control and ligated amplification sequence templates are present in the
25 same amplification reaction and use the same primers, the effect of a number of variables which can effect the efficiency of the amplification reaction is essentially nullified. Such variables included, inter alia,: (1) quality and concentration of reagents (Taq
30 DNA polymerase, primers, templates, dNTP's), (2) conditions used for denaturation, annealing and primer extension, (3) rate of change of reaction temperature and (4) priming efficiency of the oligonucleotide primers. The relative amounts of the two amplified
35 products -- i.e., ligated amplification sequence and

control template -- reflect the relative concentrations of the starting templates.

The quantitative PCR method may be generally carried out as follows:

- 5 1. A control template, e.g., a DNA sequence corresponding to nanovariant RNA, a naturally occurring template of Q β replicase (Schaffner et al., J. Mol. Biol. 117:877-907, 1977) is synthesized by automated oligonucleotide synthesis and its concentration
10 determined, e.g., by spectrophotometry or by ethidium-bromide mediated fluorescence.
2. A series of tenfold dilutions (in TE Buffer) containing from 10 ng/ml to 1 fg/ml of the control template is made and stored at -70°C until use.
- 15 3. A series of PCR amplification reactions of the free ligated amplification sequence is set up. In addition to the usual PCR ingredients, the reactions also contain about 10 μ l/reaction of the tenfold dilutions of the control template and about 10
20 μ Ci/reaction of [α -³²P] dCTP (Sp.act. 3000 Ci/mmol).
4. PCR amplification reactions are carried out for a desired number of cycles, e.g., 30-40.
5. The reaction products may then be subject to agarose gel electrophoresis and
25 autoradiography to separate the two amplified products (of different sizes). The amplified bands of the control and ligated amplification sequence are recovered from the gel using suitable techniques and radioactivity present in each band is determined by
30 counting in a liquid scintillation counter. The relative amounts of the two products are calculated based on the amount of radioactivity in each band. The amount of radioactivity in the two samples must be corrected for the differences in molecular weights of
35 the two products.

6. The reactions may be repeated using a narrower range of concentration of control template to better estimate the concentration of ligated amplification sequence.

5 In another aspect of the invention, more than the two probes i.e. a single capture/amplification probe, and a single amplification probe may be utilized. For example one or more capture/amplification probes, and one or more amplification probes, may be employed in
10 the detection and capture of the target nucleic acid, and optional amplification of the target sequences, as shown schematically in Figs. 4 and 5. According to this aspect of the present invention, the capture/amplification probes may have a 3' sequence
15 complementary to the target nucleic acid and a biotin moiety at the 5' terminus that is capable of interacting with the streptavidin coated paramagnetic beads. Alternatively, the capture/amplification probes may have a 5' sequence complementary to the target
20 nucleic acid and a biotin moiety at the 3' terminus.

Further, according to this aspect of the present invention, one or more amplification probes are utilized such that each probe contains sequences that are specifically complementary to and hybridizable with
25 the target nucleic acid. For example, the 5' end of one amplification probe, e.g. Amp-probe-2 (HCV A) in Fig. 4, contains a sequence complementary to a distinct portion in the target nucleic acid. The 3' end of the second amplification probe e.g. Amp-probe-2A
30 (HCV A) in Fig. 4, contains a specific sequence complementary to a region of the target nucleic acid that is immediately adjacent to that portion of the target hybridizable to the first amplification probe. The capture/amplification probes and the pair of
35 amplification probes hybridize with the target nucleic acid in the presence of GNSCN as described above. This

complex so formed is bound to streptavidin-coated paramagnetic beads by means of a biotin moiety on the capture/amplification probes and the complex separated from unreacted components by means of magnetic separation as above. Next, the amplification probes may be linked, for example, by a ligase enzyme. This produces a ligated amplification sequence that serves as a template for Taq DNA polymerase during amplification reaction by PCR.

10 In a particular aspect of the invention, two or more capture/amplification probes and two pairs of amplification probes are utilized for the detection of the target nucleic acid.

The use of multiple capture/amplification probes affords even better capture efficiency, permitting the capture of multiple targets with generic capture probes. This is especially desirable for multiplex PCR reactions where multiple targets within a single reaction may be detected.

20 For example, a capture/amplification probe for use in the present method may be designed to bind to the poly-A tail region of cellular mRNA, whereby all such mRNA can be isolated by a single capture-and-wash step. Subsequent PCR amplification may be designed to detect and amplify specific target pathogen or disease gene sequences from such an mRNA pool. Such genes may include, inter alia, the gene encoding the cystic fibrosis transmembrane regulator protein (CFTR) or hemoglobins or other proteins involved in genetic diseases.

30 In still another aspect of the invention, the multiple capture/amplification probes may target, for example, all strains of a particular pathogen, e.g. the Hepatitis C Virus (HCV), and amplification probes may be tailored to detect and further identify individual HCV genotypes of the pathogen (e.g. HCV).

In a further embodiment, two capture/amplification probes are utilized. e.g. as depicted in Fig. 4. This provides a total specific sequence of the capture/amplification probes complementary and hybridizable to the target nucleic acid that can be twice as long as that of a single capture/amplification probe, thereby affording an even higher capture efficiency.

The pair of capture/amplification probes, e.g. as shown in Fig. 4, may each have a 3' sequence complementary to the target nucleic acid, and a biotin moiety at its 5' terminus capable of interacting with streptavidin coated paramagnetic beads. Alternatively, the pair of capture/amplification probes may each have a 5' sequence complementary to the target nucleic acid, and a biotin moiety at its 3' terminus capable of interacting with streptavidin coated paramagnetic beads.

In still further embodiments, the present invention may utilize a single amplifiable "full length probe" and one or more capture/amplification probes as shown in Fig. 6. Further, the hybridized nucleic acid duplex, comprising of the target nucleic acid, for example, HCV RNA, and the capture/amplification probes and full length amplification probes, also referred to as amplification sequences, can be released from the magnetic beads by treating the hybridized duplex molecule with RNAase H. Alternatively, the hybridized duplex, comprising of the target nucleic acid, e.g. DNA, and the capture/amplification probes and full length amplification probes, can be released from the magnetic beads by treating the hybridized duplex molecule with appropriate restriction enzymes, as described above.

When a full length amplification probe is employed to detect a target nucleic acid sequence, the probe may

be utilized in amplification reactions such as PCR, without having to use the ligation step described above. This latter approach, in particular, simplifies the assay and is especially useful when at least 10^4 target nucleic acid molecules are available in the testing sample, so that the chances of non-specific binding in a ligation independent detection reaction are reduced. In most clinical detection assays, the target nucleic acid (such as a pathogen), is present at $>10^5$ molecules/ml. of sample, and thus would be amenable to detection and amplification by this method.

A still further aspect of the present invention utilizes one or more capture/amplification probes, each containing a biotin moiety, and a single amplification probe, also referred to as an amplification sequence, that hybridizes to the target nucleic acid and circularizes upon ligation of its free termini, as shown in Fig. 7. The amplification probe may be designed so that complementary regions (see e.g. the region shown in bold in Fig.7) of the probe that are hybridizable to the target nucleic acid sequence are located at each end of the probe (as described in Nilsson et al., 1994, Science 265:2085-2088). When the probe hybridizes with the target, its termini are placed adjacent to each other, resulting in the formation of a closed circular molecule upon ligation with a linking agent such as a ligase enzyme. This circular molecule may then serve as a template during an amplification step, e.g. PCR, using primers such as those depicted in Fig. 7.

For example, the probe, described above, can be used to detect different genotypes of a pathogen, e.g. different genotypes of HCV from serum specimens. Genotype specific probes can be designed, based on published HCV sequences (Stuyver et al., 1993, J. Gen. Virol. 74: 1093-1102), such that a mutation in the

target nucleic acid is detectable since such a mutation would interfere with (1) proper hybridization of the probe to the target nucleic acid and (2) subsequent ligation of the probe into a circular molecule.

- 5 Because of the nature of the circularized probe, as discussed below, unligated probes may be removed under stringent washing conditions.

The single, full length, ligation-dependent circularizable probe, as utilized in the method,
10 affords greater efficiency of the detection and amplification of the target nucleic acid sequence. Due to the helical nature of double-stranded nucleic acid molecules, circularized probes are wound around the target nucleic acid strand. As a result of the
15 ligation step, the probe may be covalently bound to the target molecule by means of catenation. This results in immobilization of the probe on the target molecule, forming a hybrid molecule that is substantially resistant to stringent washing conditions. This
20 results in significant reduction of non-specific signals during the assay, lower background noise and an increase in the specificity of the assay.

Moreover, in a special application, the amplification probes and/or amplification sequences as
25 described above, can be used for *in situ* LD-PCR assays. *In situ* PCR may be utilized for the direct localization and visualization of target viral nucleic acids and may be further useful in correlating viral infection with histopathological finding.

30 Current methods assaying for target viral RNA sequences have utilized RT PCR techniques for this purpose (Nuovo et al., 1993, Am. J. Surg. Pathol. 17(7): 683-690). In this method cDNA, obtained from target viral RNA by *in situ* reverse transcription, is
35 amplified by the PCR method. Subsequent intracellular localization of the amplified cDNA can be accomplished

by *in situ* hybridization of the amplified cDNA with a labelled probe or by the incorporation of labelled nucleotide into the DNA during the amplification reaction.

5 However, the RT PCR method suffers drawbacks which are overcome by the present invention. For example, various tissue fixatives used to treat sample tissues effect the crosslinking of cellular nucleic acids and proteins, e.g. protein-RNA and RNA-RNA complexes and
10 hinder reverse transcription, a key step in RT-PCR. Moreover, secondary structures in target RNA may also interfere with reverse transcription. Further, the application of multiplex PCR to RT PCR for the detection of multiple target sequences in a single cell
15 can present significant problems due to the different efficiencies of each primer pair.

 The method of the present invention utilizes one or more amplification probes and/or amplification sequences, as described above, and the LD-PCR technique
20 to locate and detect *in situ* target nucleic acid, which offers certain advantages over the RT-PCR method. First, since hybridization of the probe to target nucleic acid and subsequent amplification of the probe sequences in the eliminates the reverse transcription
25 step of the RT-PCR method, the secondary structure of the target RNA does not affect the outcome of the assay. Moreover, the crosslinking of target nucleic acids and cellular proteins due to tissue fixatives, as discussed above, does not interfere with the
30 amplification of probe sequences since there is no primer extension of the target RNA as in the RT-PCR method.

 In particular, amplification probes according to the present invention may be designed such that there
35 are common primer-binding sequences within probes detecting different genotypic variants of a target

pathogen. This enables the assay to detect multiple targets in a single sample. For example, and not by way of limitation, the assay may utilize two or more amplification probes according to this method to detect
5 HCV RNA and β -actin RNA, whereby the β -actin probe serves as an internal control for the assay.

Furthermore, the primer-binding sequences in the probe may be designed to (1) minimize non-specific primer oligomerization and (2) achieve superior primer-
10 binding and increased yield of PCR products, thereby increasing sensitivity of the assay.

Since the amplification probe that undergoes PCR circularizes after binding to target nucleic acid to become a circular molecule, multimeric products may be
15 generated during polymerization, so that amplification products are easily detectable.

An *in situ* LD-PCR assay to detect target nucleic acids in histological specimens according to the present invention utilizes a ligation dependent full
20 length amplification probe, and involves the following steps:

Sample tissue, e.g. liver, that may be frozen, or formalin-fixed and embedded in paraffin, is sectioned and placed on silane-coated slides. The sections may
25 be washed with xylene and ethanol to remove the paraffin. The sections may then be treated with a proteolytic enzyme, such as trypsin, to increase membrane permeability. The sections may be further treated with RNAase-free DNAase to eliminate cellular
30 DNA.

An amplification probe may be suspended in a suitable buffer and added to the sample sections on the slide and allowed to hybridize with the target
sequences. Preferably, the probe may dissolved in 2 x
35 SSC with 20% formamide, added to the slide, and the mixture incubated for 2 hours at 37°C for hybridization

to occur. The slide may be washed once with 2 x SSC and twice with 1 x ligase buffer before DNA ligase may be applied to the sample. Preferably, 1U/20 μ l of the ligase enzyme may be added to each slide, and the mixture incubated at 37°C for 2 hours to allow circularization of the probe. The slide may be washed with 0.2 x SSC (high stringency buffer) and 1 x PCR buffer to remove unligated probes before the next step of amplification by PCR. The PCR reaction mixture, containing the amplification primers and one or more labelled nucleotides is now added to the sample on the slide for the amplification of the target sequences. The label on the nucleotide(s) may be any signal generating moiety, including, inter alia, radioisotopes, e.g., ^{32}P or ^3H , fluorescent molecules, e.g., fluorescein and chromogenic molecules or enzymes, e.g., peroxidase, as described earlier. Chromogenic agents are preferred for detection analysis, e.g., by an enzyme linked chromogenic assay.

In a still preferred aspect, digoxinin-labelled nucleotides are utilized. In such cases the PCR product, tagged with digoxinin-labelled nucleotides is detectable when incubated with an antidigoxinin antibody-alkaline phosphatase conjugate. The alkaline phosphatase-based colorimetric detection utilizes nitroblue tetrazolium, which, in the presence of 5-Bromo-4-chloro-3-indolylphosphate, yields a purple-blue precipitate at the amplification site of the probe.

In one aspect of the present invention, the ligation and the PCR amplification step of the *in situ* LD-PCR detection method can be carried out simultaneously and at a higher temperature, by using a thermostable ligase enzyme to circularize the amplification probe.

In accordance with the present invention, further embodiments of *in situ* LD-PCR may utilize amplification

probes that are designed to detect various genotypic variants of a pathogen e.g. HCV, that are based on the known HCV sequences of these variants (Stuyver et al., 1993, J.Gen. Vir. 74: 1093-1102). For example,
5 different type-specific probes may be added together to the sample, and detection of HCV sequences and amplification of the probe sequences carried out by *in situ* LD-PCR as described above. Next, the amplified probe sequences are assayed for the presence of
10 individual variant genotypes by *in situ* hybridization with type specific internal probes that are labelled to facilitate detection.

In certain aspects of the invention, the target nucleic acid sequence may be directly detected using
15 the various amplification probes and/or amplification sequences described above, without amplification of these sequences. In such aspects, the amplification probes and/or amplification sequences may be labelled so that they are detectable.

20 Reagents for use in practicing the present invention may be provided individually or may be packaged in kit form. For example, kits might be prepared comprising one or more first, e.g., capture/amplification-1 probes and one or more second,
25 e.g., amplification-probe-2 probes, preferably also comprising packaged combinations of appropriate generic primers. Kits may also be prepared comprising one or more first, e.g., capture/amplification-1 probes and one or more second, full length, ligation-independent
30 probes, e.g., amplification-probe-2. Still other kits may be prepared comprising one or more first, e.g., capture/amplification-1 probes and one or more second, full length, ligation-dependent circularizable probes, e.g., amplification-probe-2. Such kits may preferably
35 also comprise packaged combinations of appropriate generic primers. Optionally, other reagents required

for ligation (e.g., DNA ligase) and, possibly, amplification may be included. Additional reagents also may be included for use in quantitative detection of the amplified ligated amplification sequence, e.g., control templates such as an oligodeoxyribonucleotide corresponding to nanovariant RNA. Further, kits may include reagents for the *in situ* detection of target nucleic acid sequences e.g. in tissue samples.

The arrangement of the reagents within containers of the kit will depend on the specific reagents involved. Each reagent can be packaged in an individual container, but various combinations may also be possible.

The present invention is illustrated with the following examples, which are not intended to limit the scope of the invention.

Example 1

DETECTION OF HIV-1 RNA IN A SAMPLE.

Preparation of Oligonucleotide Probes

A pair of oligodeoxyribonucleotide probes, designated Capture/Amp-probe-1 (HIV) and Amp-probe-2 (HIV), respectively for detecting the gag region of HIV-1 RNA were prepared by automated synthesis via an automated DNA synthesizer (Applied Biosystems, Inc.) using known oligonucleotide synthetic techniques. Capture/Amp-probe-1 (HIV) is an oligodeoxyribonucleotide comprising 59 nucleotides and a 3' biotin moiety, which is added by using a 3'-biotinylated nucleoside triphosphate as the last step in the synthesis. The Capture/Amp-probe-1 (HIV) used in this Example has the following nucleotide sequence (also listed below as SEQ ID NO. 1):

35 1 11 21
5' - CCATCTTCCT GCTAATTTTA AGACCTGGTA

31 41 51
ACAGGATTTC CCCGGAATT CAAGCTTGG - 3'

5 The nucleotides at positions 24-59 comprise the generic 3' end of the probe. Within this region are recognition sequences for *Sma*I (CCCGGG), *Eco*RI (GAATTC) and *Hind*III (AAGCTT) at nucleotides 41-46, 46-51 and 52-57, respectively. The 5' portion of the sequence
10 comprising nucleotides 1-23 is complementary and hybridizes to a portion of the gag region of HIV-1 RNA.

Amp-probe-2 (HIV) is a 92 nucleotide oligodeoxyribonucleotide which has the following sequence (also listed below as SEQ ID NO. 2):

15
5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG
20 51 61 71 81 91
AGGTGAGAAA ACCCCGTTAT CTGTATGTAC TGTCTTACT GG - 3'

The nucleotides at positions 71-92 comprise the 3' specific portion of this probe, complementary and
25 hybridizable to a portion of the gag region of HIV-1 RNA immediately adjacent to the portion of the gag region complementary to nucleotides 1-23 of Capture/Amp-probe-1 (HIV). Nucleotides 1-70 comprise the generic 5' portion of Amp-probe-2 (HIV).

30 Ligation of the 5' end of Capture/Amp-probe-1 (HIV) to the 3' end of Amp-probe-2 (HIV) using T_4 DNA ligase creates the ligated amplification sequence (HIV) having the following sequence (also listed below as SEQ ID NO. 3):

35
5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT
40 41 51 61 71
TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CTGTATGTAC
81 91 101 111
TGTCTTACT GGCCATCTTC CTGCTAATTT TAAGACCTGG

121 131 141 151
 TAACAGGATT TCCCCGGGAA TTCAAGCTTG G -3'

5 This ligated amplification sequence is 151 nucleotides long, which provides an ideal sized template for PCR.

The generic nucleotide sequences of the ligated amplification sequence (HIV) comprising nucleotides 116-135 (derived from nucleotides 24-43 of Capture/Amp-probe-1 (HIV)) and nucleotides 1-70 (derived from nucleotides 1-70 of Amp-probe-2 (HIV)) correspond in sequence to nucleotides 1-90 of the (-) strand of the WSI nanovariant RNA described by Schaffner *et al.*, J. Molec. Biol. 117:877-907 (1977). WSI is one of a group of three closely related 6 S RNA species, WSI, WSII and WSIII, which arose in Q β replicase reactions without added template. Schaffner *et al.* termed the three molecules, "nanovariants."

20 The 90 nucleotide long oligodeoxyribonucleotide corresponding to nucleotides 1-90 of the WSI (-) strand has the following sequence (also listed below as SEQ ID NO. 4):

25 5' - 1 11 21 31 41
 GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG
 51 61 71 81
 AGGTGAGAAA ACCCCGTTAT CCTGGTAACA GGATTCCCC - 3'

30.

Two generic oligodeoxynucleotide primers were also synthesized for use in PCR amplification of the ligated amplification sequence. Primer-1, which has a length of 21 nucleotides, is complementary to the 3' sequence of Capture/Amp-probe-1 (HIV) (nucleotides 38-58) and has the sequence (also listed below as SEQ ID NO. 5):

40 5' - 1 11
 CAAGCTTGAA TTCCCCGGGGA A - 3'

Primer-2, which has a length of 20 nucleotides, corresponds in sequence to the 5' sequence of Amp-probe-2 (HIV) (nucleotides 1-20) and has the sequence (also listed below as SEQ ID NO. 6):

5

1 11
5' - GGGTTGACCC GGCTAGATCC - 3'

10

Capture and Detection of HIV-1 RNA

Target HIV-1 RNA (100 μ l) is dissolved in an equal volume of lysis buffer comprising 5M GnSCN, 100mM EDTA, 200mM Tris-HCl (pH 8.0), 0.5% NP-40 (Sigma Chemical Co., St. Louis, MO), and 0.5% BSA in a 1.5 ml microfuge tube. Next, the 3'-biotinylated Capture/Amp-probe-1 (HIV) (SEQ ID NO. 1) and Amp-probe-2 (HIV) (SEQ ID NO. 2), together with streptavidin-coated paramagnetic beads (obtained from Promega Corp.) were added to the lysed sample in the lysis buffer. A complex comprising target RNA/Capture/Amp-probe-1 (HIV)/Amp-probe-2 (HIV)/paramagnetic beads was formed and retained on the beads. A magnetic field generated by a magnet in a microfuge tube holder rack (obtained from Promega Corp.) was applied to the complex to retain it on the side of the reaction tube adjacent the magnet to allow unbound material to be siphoned off. The complex was then washed twice with a 1.5M GnSCN buffer to remove any unbound proteins, nucleic acids, and probes that may be trapped with the complex. The magnetic field technique facilitated the wash steps. The GnSCN then was removed by washing the complex with 300mM KCl buffer (300mM KCl, 50mM Tris-HCl, pH 7.5, 0.5% Non-IDEF-40 1mM EDTA).

The two probes were then covalently joined using T₄ DNA ligase (Boehringer Mannheim) into a functional ligated amplification sequence (HIV) (SEQ ID NO. 3), which can serve as a template for PCR amplification.

The ligation reaction was carried out in the presence of a 1X ligation buffer comprising a 1:10 dilution of 10X T₄ DNA ligase ligation buffer (660mM Tris-HCl, 50mM MgCl₂, 10mM dithioerythritol, 10mM ATP - pH 7.5 at 20°C) obtained from Boehringer Mannheim.

The paramagnetic beads containing bound ligated amplification sequence (HIV) were washed with 1X T₄ DNA ligase ligation buffer and resuspended in 100 µl of 1X T₄ DNA ligase ligation buffer. 20 µl of bead suspension was removed for the ligation reaction. 2 µl T₄ DNA ligase was added to the reaction mixture, which was incubated at 37°C for 60 minutes.

For PCR amplification of the bound ligated amplification sequence (HIV), 80 µl of a PCR reaction mixture comprising Taq DNA polymerase, the two generic PCR primers (SEQ ID NOS. 5 and 6), a mixture of deoxynucleoside triphosphates and ³²P-dCTP was added to the ligation reaction. A two temperature PCR reaction was carried out for 30 cycles in which hybrid formation and primer extension was carried out at 65°C for 60 seconds and denaturation was carried out at 92°C for 30 seconds.

After 30 cycles, 10 µl of the reaction mixture was subjected to electrophoresis in a 10% polyacrylamide gel and detected by autoradiography (Fig. 3, Lane A). As a control, nanovariant DNA (SEQ ID NO. 4) was also subjected to 30 cycles of two temperature PCR, under the same conditions as for the ligated amplification sequence (HIV), electrophoresed and autoradiographed (Fig. 3, Lane B). As can be seen from Fig. 3, the amplified ligated amplification sequence (HIV) migrated in a single band (151 nucleotides) at a slower rate than the amplified nanovariant DNA (90 nucleotides). The results also indicated that unligated first and second probes were either not amplified or detected.

EXAMPLE 2DIRECT DETECTION OF HIV-1 RNA IN A SAMPLE.

The ability of the present method to directly detect the presence of HIV-1 RNA in a sample was also determined. The probes used in this Example are the same as in Example 1 (SEQ ID NOS. 1 and 2). For direct detection, Amp-probe-2 (HIV) (SEQ ID NO. 2) was labeled at its 5' end with ^{32}P by the T_4 phosphokinase reaction using ^{32}P - γ -ATP. The various reaction mixtures were as follows:

1. Streptavidin-coated paramagnetic beads, 3'-biotinylated Capture/Amp-probe-1 (HIV) (SEQ ID NO. 1), Amp-probe-2 (HIV) (SEQ ID NO. 2) 5' (^{32}P), HIV-1 RNA transcript.
2. Streptavidin-coated paramagnetic beads, 3'-biotinylated Capture/Amp-probe-1 (HIV), Amp-probe-2 (HIV) 5' (^{32}P).
3. Streptavidin-coated paramagnetic beads, Amp-probe-2 (HIV) 5' (^{32}P), HIV-1 RNA transcript.

Hybridizations, using each of the above three reaction mixtures, were carried out in 20 μl of a 1M GnSCN buffer comprising 1M GnSCN, 0.5% NP-40 (Nonidet P-40, N-lauroylsarcosine, Sigma Chemical Co., St Louis, MO), 80mM EDTA, 400mM Tris HCl (pH 7.5) and 0.5% bovine serum albumin.

The reaction mixtures were incubated at 37°C for 60 minutes. After incubation, the reaction mixtures were subjected to a magnetic field as described in Example 1 and washed (200 μl /wash) two times with 1M GnSCN buffer and three times with a 300mM KCl buffer comprising 300mM KCl, 50mM Tris-HCl (pH 7.5), 0.5% NP-40 and 1mM EDTA. The amount of ^{32}P - labeled Amp-probe-

2 (HIV) that was retained on the paramagnetic beads after washing is reported in Table 1 as counts-per-minute (CPM). The results indicate that, only in the presence of both target HIV RNA and Capture/Amp-probe-1 (HIV), is a significant amount of Amp-probe-2 retained on the beads and detected by counting in a β -scintillation counter.

TABLE 1

10

Capture of target HIV RNA with Capture/Amp-probe-1(HIV)		
Reaction Mixture	CPM (after 2 washes with 1M GNSCN)	CPM (after 3 washes with 0.3M KCl)
1.	6254	5821
2.	3351	2121
3.	3123	2021

20

EXAMPLE 3DETECTION OF MYCOBACTERIUM AVIUM-INTRACELLULAIRE
(MAI) IN PATIENT SAMPLES.

A recent paper (Boddinghaus et al., J. Clin. Microbiol. 28:1751, 1990) has reported successful identification of Mycobacteria species and differentiation among the species by amplification of 16S ribosomal RNAs (rRNAs). The advantages of using bacterial 16S rRNAs as targets for amplification and identification were provided by Rogall et al., J. Gen. Microbiol., 136:1915, 1990 as follows: 1) rRNA is an essential constituent of bacterial ribosomes; 2) comparative analysis of rRNA sequences reveals some stretches of highly conserved sequences and other stretches having a considerable amount of variability; 3) rRNA is present in large copy numbers, i.e. 10^3 to 10^4 molecules per cell, thus facilitating the development of sensitive detection assays; 4) the nucleotide sequence of 16S rRNA can be rapidly determined without any cloning procedures and the sequence of most Mycobacterial 16S rRNAs are known.

Three pairs of Capture/Amp-probe-1 and Amp-probe-2 probes are prepared by automated oligonucleotide synthesis (as above), based on the 16S rRNA sequences published by Boddinghaus et al., and Rogall et al. The first pair of probes (MYC) is generic in that the specific portions of the first and second probes are hybridizable to 16S RNA of all Mycobacteria spp; this is used to detect the presence of any mycobacteria in the specimen. The second pair of probes (MAV) is specific for the 16S rRNA of *M. avium*, and the third pair of probes (MIN) is specific for the 16S rRNA of *M. intracellulaire*. The extremely specific ligation reaction of the present method allows the differentiation of these two species at a single nucleotide level.

A. The probes that may be used for generic detection of all Mycobacter spp. comprise a first and second probe as in Example 1. The first probe is a 3' biotinylated - Capture/Amp-probe-1 (MYC), an
 5 oligodeoxyribonucleotide of 54 nucleotides in length with the following sequence (also listed below as SEQ ID NO. 7):

```

10      1      11      21      31
5' - CAGGCTTATC CCGAAGTGCC TGGTAACAGG ATTTCCCCGG
      41      51
      GAATTCAAGC TTGG - 3'
  
```

15 Nucleotides 1-18, at the 5' end of the probe are complementary to a common portion of Mycobacterial 16S rRNA, i.e., a 16S rRNA sequence which is present in all Mycobacteria spp. The 3' portion of the probe,
 20 comprising nucleotides 19-54 is identical in sequence to the 36 nucleotides comprising the generic portion of Capture/Amp-probe-1 (HIV) of Example 1.

The second probe is Amp-probe-2 (MYC), an oligodeoxyribonucleotide of 91 nucleotides in length,
 25 with the following sequence (also listed below as SEQ ID NO. 8):

```

30      1      11      21      31
5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT
      41      51      61      71
      TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CCGGTATTAG
      81      91
35      ACCCAGTTTC C - 3'
  
```

Nucleotides 71-91 at the 3' end of the probe are complementary to a common portion of 16S rRNA adjacent
 40 the region complementary to nucleotides 1-18 or Capture/Amp-probe-1 (MYC) disclosed above, common to all Mycobacteria spp. Nucleotides 1-70 at the 5' end

of the probe comprise the same generic sequence set forth for Amp-probe-2 (HIV) in Example 1.

End to end ligation of the two probes, as in Example 1, provides ligated amplification sequence
 5 (MYC), 145 nucleotides in length, for detection of all Mycobacteria spp., having the following sequence (also listed below as SEQ ID NO. 9):

```

10  5' - 1      11      21      31
      GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT
      41      51      61      71
      TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CCGGTATTAG
15      81      91      101     111
      ACCCAGTTTC CCAGGCTTAT CCCGAAGTGC CTGGTAACAG
      121     131     141
      GATTTCCTCCG GGAATTCAAG CTTGG - 3'
20

```

B. The pair of probes for specific detection of M. avium are as follows:

The first probe is a 3' biotinylated-Capture/Amp-
 25 probe-1 (MAV), an oligodeoxyribonucleotide of 56 nucleotides in length with the following sequence (also listed below as SEQ ID NO. 10):

```

30  5' - 1      11      21      31
      GAAGACATGC ATCCCGTGGT CCTGGTAACA GGATTTCCTCC
      41      51
      GCGAATTCAA GCTTGG - 3'
35

```

Nucleotides 1-20 at the 5' - end are complementary to a portion of 16S rRNA specific for M. avium. Nucleotides 21-56 comprise the same generic sequence, as above.

The second probe is Amp-probe-2 (MAV), an
 40 oligodeoxyribonucleotide of 90 nucleotides in length, with the following sequence (also listed below as SEQ ID NO. 11):


```

      1      11      21      31
5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT
      41      51      61      71
5   TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CGCTAAAGCG
      81
      CTTTCCACCA - 3'

```

10

Nucleotides 71-90 at the 3' end of the probe comprise the specific nucleotide sequence complementary to a region of 16S rRNA specific for M. avium, adjacent the specific sequence recognized by Capture/Amp-probe-1 (MAV). Nucleotides 1-70 comprise the same generic sequence as above.

15

End to end ligation of the two probes provides a 146 nucleotide long ligated amplification sequence (MAV) for detection of M. avium having the following sequence (also listed below as SEQ ID NO. 12):

20

```

      1      11      21      31
5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT
      41      51      61      71
25  TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CGCTAAAGCG
      81      91      101     111
      CTTTCCACCA GAAGACATGC ATCCCGTGGT CCTGGTAACA
30
      121     131     141
      GGATTTCCCC GGGAATTCAA GCTTGG - 3'

```

35

C. The pair of probes for specific detection of M. intracellulaire are as follows:

The first probe is a 3' - biotinylated Capture/Amp-probe-1 (MIN), an oligonucleotide of 56 nucleotides in length with the following sequence (also listed below as SEQ ID NO. 13):

40

1 11 21 31
 5' - AAAGACATGC ATCCCGTGGT CCTGGTAACA GGATTTCCCC
 41 51
 5 GCGAATTCAA GCTTGG - 3'

Nucleotides 1-20 at the 5' end are complementary to a portion of 16S rRNA specific for M. intracellulaire.

10 Nucleotides 21-56 comprise the same generic sequence as above.

The second probe is Amp-probe-2 (MIN), an oligodeoxyribonucleotide or 90 nucleotides in length, with the following sequence (also listed below as SEQ ID NO. 14):

1 11 21 31
 5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT
 41 51 61 71
 20 TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CGCTAAAGCG
 81
 25 CTTTCCACCT - 3'

Nucleotides 71-90 at the 3' end of the probe comprise the specific nucleotide sequence complementary to a region of M. intracellulaire 16S rRNA adjacent the specific sequence recognized by Capture/Amp-probe-1 (MIN).

End to end ligation of the two probes provides a 146 nucleotide long ligated amplification sequence (MIN) for detection of M. intracellulaire, having the following sequence (also listed below as SEQ ID NO. 15):

```

      1      11      21      31
5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT

      41      51      61      71
5   TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CGCTAAAGCG

      81      91     101     111
      CTTTCCACCT AAAGACATGC ATCCCGTGGT CCTGGTAACA

10  121     131     141
      GGATTTC CCC GGAATTCAA GCTTGG - 3'

```

D. In order to detect the presence of the above

15 Mycobacteria spp., patients' blood specimens are collected in Pediatric Isolator Tubes (Wampole Laboratories, NJ). The Isolator's lysis centrifugation technique enables separation of blood components, followed by lysis of leukocytes to improve recovery of

20 intracellular organisms (Shanson et al., J. Clin. Pathol. 41:687, 1988). After lysis, about 120 μ l of concentrated material is dissolved in an equal volume of the 5M GnSCN buffer of Example 1. The mixture is boiled for 30 minutes, which because of the nature of

25 mycobacterial cell walls, is required for lysis of Mycobacteria spp. The subsequent procedures (i.e., capture, ligation, PCR and detection) are the same as those employed in Example 1.

Before the PCR amplification, a direct detection

30 is made by measuring radioactivity representing ^{32}P -5'-AMP-probe-2 captured on the magnetic beads. After the unbound radioactively-labeled Amp-probe-2 is removed by extensive washing, the target 16S rRNA molecules that are present in concentrations of more than 10^6 /reaction

35 is detectable. Target 16S rRNA that cannot be detected directly is subjected to PCR amplification of the ligated amplification sequences as per Example 1. The primers for use in amplification are the same two generic primers of Example 1 (SEQ ID NOS. 5 and 6).

EXAMPLE 4DETECTION OF HCV RNA IN A SAMPLE.

Hepatitis C virus (HCV), an RNA virus, is a causative agent of post transfusion hepatitis. HCV has been found to be distantly related to flavivirus and pestivirus and thus its genome has a 5' and a 3' untranslated region (UTR) and encodes a single large open reading frame (Lee et al., J. Clin. Microbiol. 30:1602-1604, 1992). The present method is useful for detecting the presence of HCV in a sample.

A pair of oligodeoxynucleotide probes, designated Capture/Amp-probe-1 (HCV) and Amp-probe-2 (HCV), respectively, for targeting the 5' UTR of HCV RNA are prepared as in Example 1.

Capture/Amp-probe-1 (HCV), which is biotinylated at the 3' end, is a 55 nucleotide long oligodeoxyribonucleotide having the following nucleotide sequence (also listed below as SEQ ID NO. 16):

20
5' - GCAGACCACT ATGGCTCTCC CTGGTAACAG GATTTCCTCCG
41 51
25 GGAATTCAAG CTTGG - 3'

Nucleotides 1-19 at the 5' end of Capture/Amp-probe-1 (HCV) comprise a specific sequence complementary to a portion of the 5' UTR of the HCV genome. Nucleotides 20-55 at the 3' end of the probe comprise the same 36 nucleotide generic sequence as in Capture/Amp-probe-1 (HIV) of Example 1.

Amp-probe-2 (HCV) is a 90 nucleotide long oligodeoxyribonucleotide having the following nucleotide sequence (also listed below as SEQ ID NO. 17):

```

      1      11      21      31
5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT
      41      51      61      71
5   TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CCGGTGTACT
      81
    CACCGGTTCC - 3'

```

10 Nucleotides 71-90 comprise the 3' specific portion of
the probe, complementary and hybridizable to a portion
of the HCV 5' UTR immediately adjacent to the portion
of the HCV genome hybridizable to nucleotides 1-19 of
15 Capture/Amp-probe-2 (HCV). Nucleotides 1-70 comprise
the same generic sequence as in Amp-probe-2 (HIV) of
Example 1.

End to end ligation of the two probes as in
Example 1 provides a 145 nucleotide long ligated
20 amplification sequence (HCV) for detection of HCV in a
sample, having the sequence (also listed below as SEQ
ID NO. 18):

```

      1      11      21      31
25 5' - GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT
      41      51      61      71
    TCGAGTAGAG AGGTGAGAAA ACCCCGTTAT CCGGTGTACT
      81      91      101      111
30  CACCGGTTCC GCAGACCACT ATGGCTCTCC CTGGTAACAG
      121      131      141
    GATTTCCTCC GGAATTCAAG CTTGG - 3'
35

```

The ligated amplification sequence (HCV) is
amplified using a two temperature PCR reaction as in
Example 1. The PCR primers used for amplification are
40 the same two generic primers (SEQ ID NOS. 5 and 6) of
Example 1.

EXAMPLE 5USE OF MULTIPLE CAPTURE AND AMPLIFICATION
PROBES TO DETECT HCV RNA IN A SAMPLE.

A pair of amplification probes and two capture/
5 amplification probes were used to assay for and detect
HCV RNA in a sample, thereby increasing the capture
efficiency of the assay.

The capture/amplification probes Capture/Amp-
probe-1 (HCV A) (all oligomers described in this
10 Example are designated "(HCV A)" to distinguish from
the probes "(HCV)" of Example 4) having SEQ ID NO. 22
and Capture/Amp-probe-1A (HCV A) having SEQ ID NO. 23
are designed and synthesized such that their 5' termini
are biotinylated and the 3' region of the probes
15 comprises sequences complementary to and hybridizable
with sequences in the 5'UTR of HCV RNA (Fig. 4). The
generic nucleotide sequence at the 5' region of the
probes that are not hybridizable to the target nucleic
acid sequence are designed and synthesized to have
20 random sequences and a GC content of, at least, 60%,
and such that they exhibit minimal secondary structure
e.g. hairpin or foldback structures.

Capture/Amp-probe-1 (HCV A) which is biotinylated
at the 5' terminus, is a 45 nucleotide DNA oligomer,
25 such that nucleotides 5 to 45 in the 3' region, are
complementary to and hybridizable with sequences in the
5'UTR of the target HCV RNA, and that the oligomer has
the following nucleotide sequence (also listed below as
SEQ ID NO. 22):

30

5'- AAGAGCGTGA AGACAGTAGT TCCTCACAGG GGAGTGATTC
ATGGT - 3'

Capture/Amp-probe-1A (HCV A) which is also
35 biotinylated at the 5' terminus, is also a 45 nucleo-
tide DNA oligomer, such that nucleotides 5 to 45 in the

3' region are complementary to and hybridizable with sequences in the 5'UTR of HCV RNA that are immediately adjacent to the region of the 5'UTR of the HCV RNA hybridizable with Capture/Amp-probe-1 (HCV A) and such
5 that the oligomer has the following nucleotide sequence (also listed below as SEQ ID NO. 23):

5' - AAGACCCAAC ACTACTCGGC TAGCAGTCTT GCGGGGGCAC
GCCCA - 3'

10

The two amplification probes Amp-probe-2 (HCV A) and Amp-probe-2A (HCV A) each contain a nucleotide sequence complementary to and hybridizable with the conserved 5'UTR of HCV RNA.

15 Amp-probe-2 (HCV A) is a 51 nucleotide oligomer such that nucleotides 1 to 30 in the 5' region are complementary to and hybridizable with sequences in the 5'UTR of HCV RNA, and that the nucleotides 34 to 51 at the 3' terminus bind to and hybridize with PCR primer-3
20 and such that the oligomer has the following nucleotide sequence (also listed below as SEQ ID NO. 24):

5'- ACTCACCGGT TCCGCAGACC ACTATGGCTC GTTGTCTGTG
TATCTGCTAA C - 3'

25

Amp-probe-2A (HCV A) is a 69 nucleotide oligomer such that nucleotides 40 to 69 in the 3' region are complementary to and hybridizable with sequences in the 5'UTR of HCV RNA genome immediately adjacent to the
30 portion of the HVC RNA genome hybridizable to nucleotides 1-30 of Amp-probe-2 (HCV A) and such that the nucleotides 1 to 18 at the 5' terminus bind to and hybridize with PCR primer-4 and such that nucleotides 19 to 36 at the 5' terminus bind to and hybridize with
35 PCR primer-5, and such that the oligomer has the

following nucleotide sequence (also listed below as SEQ ID NO. 25):

5' - CAAGAGCAAC TACACGAATT CTCGATTAGG TTAGTGCAGA
5 GGACCCGGTC GTCCTGGCAA TTCCGGTGT - 3'

End to end ligation of the two probes provides a 120 nucleotide ligated product, the ligation-amplification sequence (HCV A) that serves as a
10 detectable sequence for HCV as well as a template for amplification reactions, and has the sequence (also listed below as SEQ ID NO. 26):

5'- CAAGAGCAAC TACACGAATT CTCGATTAGG TTAGTGCAGA
15 GGACCCGGTC GTCCTGGCAA TTCCGGTGTA CTCACCGGTT
CCGCAGACCA CTATGGCTCG TTGTCTGTGT ATCTGCTAAC - 3'

Primer-3, used for the first series of PCR amplification of the ligated amplification sequence, SEQ ID NO. 26 (HCV A), and which has a length of 18
20 nucleotides, is complementary to sequence comprising nucleotides 34 to 51 at the 3' terminus of Amp-probe-2 (HCV A), and is, therefore, also complementary to the sequence comprising nucleotides 103 to 120 of the
25 ligated amplification sequence, SEQ ID NO. 26 (HCV A), and has the sequence (also listed below as SEQ ID NO. 27):

5'- GTTAGCAGAT ACACAGAC - 3'
30

Primer-4, used for the first series of PCR amplification of the ligated amplification sequence (HCV A), SEQ ID NO. 26, and which has a length of 18
35 nucleotides, is complementary to the sequence comprising nucleotides 1-18 at the 5' terminus of the Amp-probe-2A (HCV A), and is, therefore, also

complementary to the sequence comprising nucleotides 1 to 18 of the ligated amplification sequence, SEQ ID NO. 26 (HCV A), and has the sequence (also listed below as SEQ ID NO. 28):

5

5' - CAAGAGCAAC TACACGAA -3'

Primer-5, a DNA oligomer of 18 nucleotides is used for the second series of PCR amplification of the
10 ligated amplification sequence (HCV A), SEQ ID NO. 26, such that the primer is complementary to the sequence comprising nucleotides 19-36 of the Amp-probe-2A (HCV A), and is, therefore, also hybridizable to the
15 sequence comprising nucleotides 19-36 of the ligated amplification sequence SEQ ID NO. 26 (HCV A), and has the sequence (also listed below as SEQ ID NO. 29):

5' - TTCTCGATTA GGTACTG - 3'

20 The assay utilizing the above probes and primers was used to detect HCV RNA in 24 human serum samples that were stored at -70°C until use. For the assay, 180 µl serum sample was added to concentrated lysis buffer (prepared by condensing 250µl of the lysis
25 solution containing 5M GnSCN, 0.5% bovine serum albumin, 80mM EDTA, 400mM Tris HCl (pH 7.5), and 0.5% Nonidet P-40 so that the mixture of serum and lysis buffer would have a final concentration of 5M GnSCN) mixed well and incubated for 1 hour at 37°C to release
30 the target RNA from HCV particles. 80µl of the lysis mixture was then transferred to 120µl of hybridization buffer [0.5% bovine serum albumin, 80mM EDTA, 400 mM Tris-Hcl (pH 7.5), 0.5% Nonidet-P40] with 10¹⁰ molecules each of amplification probes, Amp-probe-2 (HCV A) and
35 Amp-probe-2A (HCV A) oligomers, and 10¹¹ molecules each of capture/amplification probes, Capture/Amp-probe-1

(HCV A) and Capture/Amp-probe-1A (HCV A). The addition of the hybridization buffer reduced the concentration of the guanidium isothiocyanate (GnSCN) from 5M to 2M to allow the hybridization to occur. The mixture was
5 incubated at 37°C for 1 hour to let the various probes hybridize with the target RNA, whereupon 30 μ l of streptavidin-coated paramagnetic beads (Promega) were added to the hybridization mixture before incubation at 37° C for 20 minutes to allow ligand binding. Next,
10 the beads were washed with 150 μ l of 2M GnSCN to eliminate any free probes, proteins, extraneous nucleic acids and potential PCR inhibitors from the hybridization mixture; this was followed by the removal of the GnSCN by washing twice with 150 μ l ligase buffer
15 [66mM Tris-HCl (pH 7.5) 1mM DTT, 1mM ATP, 0.5% Nonidet P-40 and 1mM MnCl₂]. At each wash-step, the magnetic separation of the bound complex from the supernatant was effected by the magnetic field technique described in Example 1.

20 The amplification probes, Amp-probe-2 (HCV A) and Amp-probe-2A (HCV A), bound to target RNA were then covalently joined to create the ligated amplification sequence (HCV A) that was utilized as a template for PCR amplification. The hybrid complex was resuspended
25 in 20 μ l ligase buffer with 5 units of T₄ DNA ligase (Boehringer) and incubated for 1 hour at 37°C for the ligation reaction. For the subsequent PCR reaction referred to hereafter as the "first PCR reaction", 10 μ l of the ligated mixture, including the beads, was added
30 to 20 μ l of PCR mixture [0.06 μ M each of Primer-3 and Primer-4, 1.5 Units Taq DNA Polymerase, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.5 mM MgCl₂, 10mM Tris-HCl (pH 8.3) 50mM KCl] and the mixture incubated at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1
35 minute, for 35 cycles. After the first PCR reaction, 5 μ l of the product was transferred to a second PCR

mixture [same components as the first PCR mixture except that Primer-4 was substituted with Primer-5] for "the second PCR reaction" (a semi-nested PCR approach to increase the sensitivity of the assay) carried out under the same conditions as the first PCR reaction. 10 μ l of the products of the second reaction were electrophoresed on a 6% polyacrylamide gel, stained with ethidium bromide and visualized under ultraviolet light.

10 To establish the sensitivity and the specificity of the method, 10-fold serial dilutions of synthetic HCV RNA in HCV-negative serum were assayed according to the protocol described above, so that the concentration of HCV RNA ranged from 10 to 10^7 molecules/reaction. 15 After ligation and amplification, the PCR products were separated by polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized under ultra violet light. The results, shown in Fig. 8, clearly indicate the specificity of the method. In the 20 absence of HCV RNA there is no signal, indicating that probes must capture the target RNA in order to generate a PCR product. As few as 100 molecules of HCV RNA/sample were detectable with the semi-nested PCR method (Fig. 8), indicating that the sensitivity of the method 25 is at least comparable to that of conventional RT-PCR (Clementi et al., 1993, PCR 2: 191-196).

Further, relative amounts of the PCR product represented by the intensity of the bands as visualized in Fig. 8, were proportional to the quantity of the 30 target RNA (HCV RNA transcripts). Therefore, the assay is quantitative over, at least, a range of 10^2 to 10^5 target molecules.

To determine the increased capture efficiency afforded by two capture probes, 32 P-labelled target HCV 35 RNA was assayed for capture and retention on paramagnetic beads using Capture/Amp-probe-1 (HCV A) or

Capture/Amp-probe-1A (HCV A) or both. The capture was estimated by the amount of radioactivity retained on the paramagnetic beads after extensive washes with 2M-GnSCN buffer and the ligase buffer. Results showed that 25.7% of labelled HCV RNA was retained on the beads when captured by Capture/Amp-probe-1 (HCV A) alone, 35.8% retained with Capture/Amp-probe-1A (HCV A) alone and 41.5% of the target RNA was retained when both the capture probes were used. Therefore the double-capture method was more efficient than the use of a single capture probe.

EXAMPLE 6

USE OF MULTIPLE CAPTURE AND AMPLIFICATION PROBES TO DETECT HIV-1 RNA IN A SAMPLE.

An alternative approach to that set forth in Example 1 uses a capture/amplification probe and a pair of amplification probes to detect the presence of HIV-1 RNA. Capture/Amp-probe-1 (HIV), SEQ ID NO. 1 and a pair of amplification probes Amp-probe-2 (HIV A) (all oligomers described in this Example are designated "(HIV A)" to distinguish from the probes "(HIV)" of Example 1) (SEQ ID NO. 19) and Amp-probe-2A (HIV A), (SEQ ID NO. 20), are utilized such that the generic nucleotide sequences of the ligated amplification sequence (HIV A) (SEQ ID NO. 21) comprising nucleotides 1-26 derived from nucleotides 1-26 of Amp-probe-2 (HIV A) and nucleotides 86-112 derived from nucleotides 40-65 of Amp-probe-2A (HIV A) are designed and synthesized to have random sequences and a GC content of, at least, 60%, and such that they exhibit minimal secondary structure e.g. hairpin or foldback structures.

Amplification probe Amp-probe-2 (HIV A) is a 47 nucleotide DNA oligomer such that nucleotides 27 to 47 in the 3' region, are complementary to and hybridizable with sequences in the gag region of the target HIV-1

RNA, and that the oligomer has the following nucleotide sequence (also listed below as SEQ ID NO. 19):

5' - GGTGAAATTG CTGCCATTGT CTGTATGTTG TCTGTGTATC
5 TGCTAAC - 3'

Amplification probe Amp-probe-2A (HIV A) is a 65 nucleotide DNA oligomer such that nucleotides 1 to 39 in the 5' region, are complementary to and hybridizable with sequences in the gag region of the target HIV-1 RNA, immediately adjacent to the portion of the HIV-1 RNA genome hybridizable to nucleotides 27-47 of the Amp-probe-2 (HIV A) and that the oligomer has the following nucleotide sequence (also listed below as SEQ ID NO. 20):

5' - CAAGAGCAAC TACACGAATT CTCGATTAGG TTA CTGCAGC
AACAGGCGGC CTTAACTGTA GTACT -3'

20 End to end ligation of the two amplification probes provides a 112 nucleotide ligated amplification sequence (HIV A) such that the sequence serves as a detectable sequence for HIV-1 RNA as well as a template for amplification reactions, and has the following
25 sequence (also known as SEQ ID NO. 21)

5' - GGTGAAATTG CTGCCATTGT CTGTATGTTG TCTGTGTATC
TGCTAACCAA GAGCAACTAC ACGAATTCTC GATTAGGTTA
CTGCAGCAAC AGGCGGCCTT AACTGTAGTA CT -3'

30 Further, the capture, detection and optional amplification of the captured ligation product in order to assay for HIV RNA is carried out as described in Example 5. The PCR primers used for amplification are the same primers- 3, 4 and 5 (SEQ ID NOS. 27, 28 and
35 29) of Example 5.

EXAMPLE 7

USE OF SEPARATE CAPTURE/AMPLIFICATION
PROBES AND A LIGATION INDEPENDENT, SINGLE
AMPLIFICATION PROBE TO DETECT HCV RNA IN A SAMPLE.

5 The assay employs a single ligation independent
amplification probe and two capture/amplification
probes to detect HCV RNA in a sample.

 The capture/amplification probes Capture/Amp-
probe-1 (HCV A) and Capture/Amp-probe-1A (HCV A) used
10 in this method are the same as described in Example 5.

 The amplification probe, Amp-probe-2 (HCV B) (all
oligomers described in this Example are designated
"(HCV B)" to distinguish from the probes "(HCV)" of
Example 4), SEQ ID NO. 30, is a 100 nucleotide DNA
15 molecule such that the sequence represented by
nucleotides 39 to 79 in the central region of the
oligomer is complementary to and hybridizable to a
region in the 5' UTR of the HCV RNA (Fig. 6), and that
the sequences spanning nucleotides 1-38 in the
20 5' terminus and by nucleotides 80-100 in the 3' terminus
are designed and synthesized such that they have random
sequences and a GC content of, at least, 60%, and such
that they exhibit minimal secondary structure e.g.
hairpin or foldback structures. Amp-probe-2 (HCV B),
25 also referred to as amplification sequence, has the
following sequence (also listed below as SEQ ID NO.
30):

 5' - CAAGAGCAAC TACACGAATT CTCGATTAGG TTAGTGCAGC
 GTCCTGGCAA TTCCGGTGTA CTCACCGGTT CCGCAGACCG
30 TTGTCTGTGT ATCTGCTAAC -3'

 The capture, detection and the optional amplifica-
tion of the probe sequences was carried out as
described in Example 5, except that primers -3 and -4,
35 only, were utilized in a single PCR amplification step,

the second PCR step was omitted, and that the ligation step was omitted.

EXAMPLE 8

5 USE OF SEPARATE CAPTURE/AMPLIFICATION
 PROBES AND AN SINGLE, AMPLIFIABLE, LIGATION
 DEPENDENT PROBE TO DETECT HCV RNA IN A SAMPLE.

 The method in this Example employs the two
capture/amplification probes Capture/Amp-probe-1 (HCV
10 A) and Capture/Amp-probe-1A (HCV A) described in
Example 5 and a single amplification probe, Amp-probe-2
(HCV C) (all oligomers described in this Example are
designated "(HCV C)" to distinguish from the probes
"(HCV)" of Example 4) that hybridizes to the target
15 nucleic acid and circularizes upon ligation of its free
termini as shown in Fig. 7.

 Amp-probe-2 (HCV C) is a 108 nucleotide
amplification probe, also referred to as an
amplification sequence, such that nucleotides 1-26 in
20 the 5' terminus of the oligomer are complementary to
and hybridizable to a sequence in the 5'UTR of the
target HCV RNA (indicated by (a) in Fig. 7) and such
that nucleotides 83-108 at the 3' terminus of the
oligomer are complementary to and hybridizable to a
25 sequence in the 5'UTR of the target HCV RNA (indicated
by (b) in Fig. 7). Moreover, when the probe hybridizes
with the target HCV RNA, the 3' and 5' termini of the
probe are placed immediately adjacent to each other
(Fig. 7), resulting in the formation of a closed
30 circular molecule upon ligation with a linking agent,
such as DNA ligase. The sequence of Amp-probe-2 (HCV
C) is given as follows (also listed as SEQ ID NO. 31):

5' - CCTTTCGCGA CCCAACACTA CTCGGCTGTC TGTGTATCTG
CTAACCAAGA GCAACTACAC GAATTCTCGA TTAGGTTACT
GCGCACCCTA TCAGGCAGTA CCACAAGG -3'

5 Primer-3 (SEQ ID NO. 27), used for the first
series of PCR amplification of the ligated and circu-
larized Amp-probe-2 (HCV C), is an 18 nucleotide long
oligomer that is complementary to the sequence
comprising nucleotides 27 to 45 of Amp-probe-2 (HCV C).

10 Primer-4 (SEQ ID NO. 28), also used for the first
series of PCR amplification of the ligated and
circularized Amp-probe-2, is a 18 nucleotide long
oligomer that is complementary to the sequence
comprising nucleotides 46 to 63 of Amp-probe-2 (HCV C).

15 The hybridization of the two capture/amplification
probes and the amplification probe to target HCV RNA,
circularization of the amplification probe upon
ligation of its termini and amplification of the probe
sequences was carried out as described in Example 5,
20 except that primers -3 and -4, only, were utilized in a
single PCR amplification step, the second PCR step was
omitted, and that Amp-probe-2 (HCV C) (SEQ ID NO. 31)
was substituted for the pair of amplification probes,
Amp-probe-2 (HCV A) (SEQ ID NO. 24) and Amp-probe-2A
25 (HCV A) (SEQ ID NO. 25) utilized in Example 5.

To establish the sensitivity and the specificity
of the method, 10-fold serial dilutions of synthetic
HCV RNA in HCV-negative serum were assayed according to
the method to provide standard concentrations of HCV
30 RNA ranging from 10^3 to 10^7 molecules/sample. After
ligation and amplification, the PCR products were
separated by polyacrylamide gel electrophoresis,
stained with ethidium bromide and visualized under
ultra-violet light.

35 The results, (Fig. 9, (-): control, no sample),
indicate the specificity of the method. The assay is

highly specific; in the absence of target HCV RNA there is no visible signal, indicating that probes must capture the target RNA in order to generate a PCR product. As seen in Fig. 9, as few as 10^4 molecules of HCV RNA/sample were clearly detectable.

Further, relative amounts of the PCR product, represented by the intensity of the bands (Fig. 9), were proportional to the quantity of the target RNA (HCV RNA transcripts). Therefore, the assay is significantly quantitative at least over a range of 10^4 to 10^7 target molecules.

EXAMPLE 9

DETECTION OF HCV TARGET SEQUENCES IN TISSUE SAMPLE USING IN SITU LD-PCR ASSAY.

Sample tissue, e.g. liver, that is frozen, or formalin-fixed and embedded in paraffin, is sectioned (4-10 μ l sections), and placed on silane-coated slides. The sections can be washed with xylene and ethanol to remove the paraffin. The sections are then treated with a proteolytic enzyme such as trypsin (2mg/ml) at 37°C for 4-12 minutes to increase membrane permeability. The sections may be further treated with RNAase-free DNAase to eliminate cellular DNA. Next, Amp-probe-2 (HCV C), (SEQ ID NO. 31), described in Example 8, is dissolved in 2 x SSC with 20% formamide, (10^{10} molecules/100 μ l), added to the slide and the mixture incubated for 2 hours at 37°C for hybridization to occur. The slide is washed once with 2 x SSC and twice with 1 x ligase buffer before the ligation step. Next, 1U/20 μ l of DNA ligase is added to each slide, and the mixture incubated at 37°C for 2 hours to allow circularization of the hybridized probe. The slide is washed with 0.2 x SSC (high stringency buffer) and 1 x PCR mixture (the same as described in Example 5) to remove unligated probe before the sample is subjected

to amplification by PCR. Next, 10-20 μ l of the PCR reaction mixture, prepared as described in Example 5, and containing the amplification primers, e.g., primers 3 and 4 (SEQ ID NOS. 27 and 28) is added to the sample
5 on the slide for the amplification of the probe sequences.

The PCR product, labelled with the digoxinin, is detectable when incubated with antidigoxinin antibody-alkaline phosphatase conjugate (Boehringer Mannheim).
10 The alkaline phosphatase-based colorimetric detection method uses the the chromagen nitroblue tetrazolium, which in the presence of 5-Bromo-4-chloro-3-indolylphosphate, yields a purple-blue precipitate at the amplification site of the probe.

Various publications are cited herein, the contents of which are hereby incorporated by reference in their entirety.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Zhang, David Y.
- (ii) TITLE OF INVENTION: LIGATION-DEPENDENT AMPLIFICATION FOR THE DETECTION OF INFECTIOUS PATHOGENS AND ABNORMAL GENES
- (iii) NUMBER OF SEQUENCES: 31
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Brumbaugh, Graves, Donohue & Raymond
 - (B) STREET: 30 Rockefeller Plaza
 - (C) CITY: New York
 - (D) STATE: NY
 - (E) COUNTRY: USA
 - (F) ZIP: 10112-0228
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Seide, Rochelle K.
 - (B) REGISTRATION NUMBER: 32,300
 - (C) REFERENCE/DOCKET NUMBER: 29545-A-PCT
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 212-408-2626
 - (B) TELEFAX: 212-765-2519

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 59 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (ix) FEATURE:
 - (A) NAME/KEY: misc feature
 - (B) LOCATION: 1..59

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCATCTTCCT GCTAATTTTA AGACCTGGTA ACAGGATTTC CCCGGGAATT CAAGCTTGG

59

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 92 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..92

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA	60
ACCCCGTTAT CTGTATGTAC TGTTTTACT GG	92

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 151 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..151

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA	60
ACCCCGTTAT CTGTATGTAC TGTTTTACT GGCCATCTTC CTGCTAATTT TAAGACCTGG	120
TAACAGGATT TCCC CGGGAA TTCAAGCTTG G	151

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..90

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA	60
ACCCCGTTAT CCTGGTAACA GGATTCCCC	90

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: misc feature
 - (B) LOCATION: 1..21

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAAGCTTGAA TTCCCGGGGA A

21

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: misc feature
 - (B) LOCATION: 1..20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGGTTGACCC GGCTAGATCC

20

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 54 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: misc feature
 - (B) LOCATION: 1..54

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAGGCTTATC CGAAGTGCC TGGTAACAGG ATTTCCCCGG GAATCAAGC TTGG

54

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 91 base pairs

-68-

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..91

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA 60
ACCCCGTTAT CCGGTATTAG ACCCAGTTTC C 91

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 145 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..145

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA 60
ACCCCGTTAT CCGGTATTAG ACCCAGTTTC CCAGGCTTAT CCCGAAGTGC CTGGTAACAG 120
GATTTCCCCG GGAATTCAAG CTGG 145

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 56 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..56

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAAGACATGC ATCCCGTGGT CCTGGTAACA GGATTTCCCC GGGAATTCAA GCTTGG 56

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 90 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 (A) NAME/KEY: misc feature
 (B) LOCATION: 1..90

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA    60
ACCCCGTTAT CGCTAAAGCG CTTTCCACCA                                     90
```

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 146 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 (A) NAME/KEY: misc feature
 (B) LOCATION: 1..146

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA    60
ACCCCGTTAT CGCTAAAGCG CTTTCCACCA GAAGACATGC ATCCCGTGGT CCTGGTAACA    120
GGATTTCCCC GGAATTCAA GCTTGG                                           146
```

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 56 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 (A) NAME/KEY: misc feature
 (B) LOCATION: 1..56

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
AAAGACATGC ATCCCGTGGT CCTGGTAACA GGATTTCCCC GGAATTCAA GCTTGG    56
```

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 90 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 1..90

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA 60
ACCCCGTTAT CGCTAAAGCG CTTTCCACCT 90

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 146 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 1..146

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA 60
ACCCCGTTAT CGCTAAAGCG CTTTCCACCT AAAGACATGC ATCCCGTGGT CCTGGTAACA 120
GGATTTCGCC GGAATTCAA GCTTGG 146

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
(A) NAME/KEY: misc feature
(B) LOCATION: 1..55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCAGACCACT ATGGCTCTCC CTGGTAACAG GATTTCCTCCG GGAATTCAAG CTTGG

55

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 90 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: misc feature
 - (B) LOCATION: 1..90

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA

60

ACCCCGTTAT CCGGTGTACT CACCGGTTCC

90

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 145 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: misc feature
 - (B) LOCATION: 1..145

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGGTTGACCC GGCTAGATCC GGGTGTGTCC TCTCTAACTT TCGAGTAGAG AGGTGAGAAA

60

ACCCCGTTAT CCGGTGTACT CACCGGTTCC GCAGACCACT ATGGCTCTCC CTGGTAACAG

120

GATTTCCTCCG GGAATTCAAG CTTGG

145

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 47 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- (ix) FEATURE:
 - (A) NAME/KEY: misc feature
 - (B) LOCATION: 1..47

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGTGAAATTG CTGCCATTGT CTGTATGTTG TCTGTGTATC TGCTAAC

47

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 65 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..65

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CAAGAGCAAC TACACGAATT CTCGATTAGG TTACTGCAGC AACAGGCCGC CTTAACTGTA

60

GTACT

65

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 112 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..112

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGTGAAATTG CTGCCATTGT CTGTATGTTG TCTGTGTATC TGCTAACCAA GAGCAACTAC

60

ACGAATTCTC GATTAGGTTA CTGCAGCAAC AGGCGGCCTT AACTGTAGTA CT

112

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AAGAGCGTGA AGACAGTAGT TCCTCACAGG GGAGTGATTC ATGGT

45

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AAGACCCAAC ACTACTCGGC TAGCAGTCTT GCGGGGGCAC GCCCA

45

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..51

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ACTCACCGGT TCCGCAGACC ACTATGGCTC GTTGTCTGTG TATCTGCTAA C

51

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 69 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..69

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CAAGAGCAAC TACACGAATT CTCGATTAGG TTACTGCAGA GGACCCGGTC GTCCTGGCAA

60

TTCCGGTGT

69

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 120 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..120

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CAAGAGCAAC TACACGAATT CTCGATTAGG TTACTGCAGA GGACCCGGTC GTCCTGGCAA 60

TTCCGGTGTA CTCACCGGTT CCGCAGACCA CTATGGCTCG TTGTCTGTGT ATCTGCTAAC 120

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTTAGCAGAT ACACAGAC 18

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CAAGAGCAAC TACACGAA 18

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..18

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTCTCGATTA GGTTACTG

18

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..100

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CAAGAGCAAC TACACGAATT CTCGATTAGG TTA CTGTCAGC GTCCTGGCAA TTCCGGTGTGTA

60

CTCACC GGTT CCGCAGACCG TTGTCTGTGT ATCTGCTAAC

100

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 108 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: misc feature
- (B) LOCATION: 1..108

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CCTTTCGCGA CCCAACACTA CTCGGCTGTC TGTGTATCTG CTAACCAAGA GCAACTACAC

60

GAATTCTCGA TTAGGTTACT GCGCACCCTA TCAGGCAGTA CCACAAGG

108

CLAIMS

- 1 1. A method for detecting a target nucleic acid in a
2 clinical sample comprising the steps of:
 - 3 (a) contacting the target nucleic acid in
4 the sample in a reaction vessel under conditions
5 that allow nucleic acid hybridization between
6 complementary sequences in nucleic acids with a
7 first and second oligonucleotide probe in the
8 presence of paramagnetic particles coated with a
9 ligand binding moiety, said first and second
10 probes comprising a first capture/amplification
11 probe containing a ligand at the 3' end thereof,
12 said ligand being able to bind to and form an
13 affinity pair with the ligand binding moiety
14 coated onto the paramagnetic particles, and
15 comprising a 3' nucleotide sequence that is
16 neither complementary nor hybridizable to a
17 nucleotide sequence in the target nucleic acid and
18 a 5' nucleotide sequence that is complementary and
19 hybridizable to a nucleotide sequence in the
20 target nucleic acid, and a second amplification
21 probe comprising a 3' nucleotide sequence that is
22 complementary and hybridizable to a nucleotide
23 sequence in the target nucleic acid immediately
24 adjacent to the target nucleic acid nucleotide
25 sequence that is complementary to the 5'
26 nucleotide sequence of the first probe and a 5'
27 nucleotide sequence that is neither complementary
28 nor hybridizable to a nucleotide sequence in the
29 target nucleic acid, such that a complex is formed
30 comprising the target nucleic acid, first and
31 second probes and the paramagnetic particles,
32 wherein the first and second probes hybridize to
33 the complementary nucleotide sequences in the
34 target nucleic acid and the first probe is bound

35 to the paramagnetic particles through the binding
36 of the ligand on the first probe to the ligand
37 binding moiety on the paramagnetic particles;
38 (b) separating the complex from unbound
39 reactants and washing the complex;
40 (c) ligating the first and second probes
41 with a ligating agent that joins nucleotide
42 sequences such that a contiguous ligated
43 amplification sequence is formed;
44 (d) separating the target nucleic acid from
45 the complex under conditions such that the ligated
46 amplification sequence remains bound to the
47 paramagnetic particles;
48 (e) washing the bound ligated amplification
49 sequence to remove the separated target nucleic
50 acid and resuspending the bound ligated
51 amplification sequence in a salt solution for
52 stabilization thereof; and
53 (f) detecting the ligated amplification
54 sequence, wherein the detection thereof indicates
55 the presence of the target nucleic acid in the
56 clinical sample.

1 2. The method of Claim 1 further comprising the step
2 of amplifying the ligated amplification sequence
3 under conditions, wherein any unligated first and
4 second probes are not amplified, said step being
5 performed after the washing step (e) and before
6 the detecting step (f), such that the amplified
7 ligated amplification sequence is detected.

1 3. The method according to Claim 1, wherein one or
2 more capture/amplification probes are used, each
3 probe having a ligand bound to the 3' or 5' end
4 thereof and each having a region of nucleotide
5 sequence that is complementary to and a region

6 that is non-complementary to the target nucleic
7 acid, wherein the ligand is bound to the non-
8 complementary region of the probe, and wherein a
9 pair of amplification probes is used, each
10 amplification probe having a region of sequence
11 that is complementary to and a region that is non-
12 complementary to the target nucleic acid, wherein
13 the complementary sequences of the amplification
14 probes hybridize to target nucleic acid sequences
15 immediately adjacent one another and wherein the
16 amplification probes, but not the
17 capture/amplification probes are ligated together.

1 4. The method according to Claim 2, wherein one or
2 more capture/amplification probes are used, each
3 probe having a ligand bound to the 3' or 5' end
4 thereof and each having a region of nucleotide
5 sequence that is complementary to and a region
6 that is non-complementary to the target nucleic
7 acid, wherein the ligand is bound to the non-
8 complementary region of the probe, and wherein a
9 pair of amplification probes is used, each
10 amplification probe having a region of sequence
11 that is complementary to and a region that is non-
12 complementary to the target nucleic acid, wherein
13 the complementary sequences of the amplification
14 probes hybridize to target nucleic acid sequences
15 immediately adjacent one another and wherein the
16 amplification probes, but not the
17 capture/amplification probes are ligated together.

1 5. The method to Claim 3, wherein one or more pairs
2 of amplification probes are used.

1 6. The method to Claim 4, wherein one or more pairs
2 of amplification probes are used.

1 7. The method according to Claim 1, wherein one or
2 more capture/amplification probes are used, each
3 probe having a ligand bound to the 3' or 5' end
4 thereof and each having a region of nucleotide
5 sequence that is complementary to and a region
6 that is non-complementary to the target nucleic
7 acid, wherein the ligand is bound to the non-
8 complementary region of the probe, and wherein a
9 single amplification sequence is used, said
10 amplification sequence having 3' and 5' regions
11 that are non-complementary to the target nucleic
12 acid sequence flanking a region that is
13 complementary to the target nucleic acid, such
14 that the capture/amplification probes are not
15 ligated to the amplification sequence after
16 hybridization.

1 8. The method according to Claim 2, wherein one or
2 more capture/amplification probes are used, each
3 probe having a ligand bound to the 3' or 5' end
4 thereof and each having a region of nucleotide
5 sequence that is complementary to and a region
6 that is non-complementary to the target nucleic
7 acid, wherein the ligand is bound to the non-
8 complementary region of the probe, and wherein a
9 single amplification sequence is used, said
10 amplification sequence having 3' and 5' regions
11 that are not complementary to the target nucleic
12 acid sequence flanking a region that is
13 complementary to the target nucleic acid, such
14 that the capture/amplification probes are not
15 ligated to the amplification sequence after
16 hybridization.

1 9. The method according to Claim 1, wherein one or
2 more capture/amplification probes are used, each

3 having a ligand bound to the 3' or 5' end thereof
4 and each having a region of nucleotide sequence
5 that is complementary to and a region that is non-
6 complementary to the target nucleic acid, wherein
7 the ligand is bound to the non-complementary
8 region of the probe, and wherein a single
9 amplification sequence is used, said amplification
10 sequence having 3' and 5' regions that are
11 complementary to adjacent sequences in the target
12 nucleic acid, said 3' and 5' regions flanking a
13 region of the amplification sequence that is non-
14 complementary to the target nucleic acid, wherein
15 the 3' and 5' ends of the amplification probe, but
16 not the capture/amplification probes, are ligated
17 together, such that upon ligation the hybridized
18 amplification probe is circularized.

1 10. The method according to Claim 2, wherein one or
2 more capture/amplification probes are used, each
3 having a ligand bound to the 3' or 5' end thereof
4 and each having a region of nucleotide sequence
5 that is complementary to and a region that is non-
6 complementary to the target nucleic acid, wherein
7 the ligand is bound to the non-complementary
8 region of the probe, and wherein a single
9 amplification probe is used, said probe having 3'
10 and 5' regions that are complementary to adjacent
11 sequences in the target nucleic acid, said 3' and
12 5' regions flanking a region of the amplification
13 probe that is non-complementary, wherein the 3'
14 and 5' ends of the amplification probe, but not
15 the capture/amplification probes, are ligated
16 together, such that upon ligation the hybridized
17 amplification probe is circularized.

- 1 11. The method according to any of Claims 1-10,
2 wherein the target nucleic acid is specific for a
3 pathogenic microorganism or virus.
- 1 12. The method according to any of Claims 1-10,
2 wherein the target nucleic acid is a normal or
3 abnormal human gene.
- 1 13. The method according to any of Claims 1-10,
2 wherein the target nucleic acid is an RNA or DNA
3 molecule.
- 1 14. The method according to any of Claims 1-10,
2 wherein the probes are oligodeoxyribonucleotides
3 or oligoribonucleotides.
- 1 15. The method according to any of Claims 1-10,
2 wherein the ligand is selected from the group
3 consisting of biotin, antigens, haptens,
4 antibodies heavy metal derivatives, and
5 polynucleotides including poly dG, poly dT, poly
6 dC, poly dA and poly U.
- 1 16. The method according to any of Claims 1-10,
2 wherein the ligand binding moiety is selected from
3 the group consisting of streptavidin, avidin,
4 antibodies, antigens, thiogroups and
5 polynucleotides including poly dC, poly dA, poly
6 dG, poly dT and poly U.
- 1 17. The method according to any of Claims 1-10,
2 wherein the ligand is biotin.
- 1 18. The method according to any of Claims 1-10,
2 wherein the ligand binding moiety is streptavidin.

- 1 19. The method according to any of Claims 1-10 further
2 comprising effecting lysis of the sample prior to
3 formation of the complex.
- 1 20. The method of Claim 19, wherein the sample is
2 mixed with a lysis buffer containing a chaotropic
3 agent and a detergent.
- 1 21. The method of Claims 1, 3, 5, 7 and 9, wherein the
2 amplification probe or sequence contains a signal
3 generating moiety at the 5' or 3' end thereof.
- 1 22. The method of Claim 21, wherein the signal
2 generating moiety is selected from the group
3 consisting of radioisotopes, chromogenic
4 substrates and fluorogenic substrates.
- 1 23. The method of Claim 21, wherein the signal
2 generating moiety is ³²P.
- 1 24. The method of Claim 21, wherein the step of
2 detecting the amplification sequence comprises
3 measuring the amount of signal generating moiety
4 retained by the paramagnetic particles.
- 1 25. The method of Claim 24, wherein the signal
2 generating moiety is ³²P and detection is performed
3 using a scintillation counter.
- 1 26. The method of any of Claims 1-6, 9 and 10, wherein
2 the ligating agent is an enzyme or a chemical
3 agent.
- 1 27. The method of Claim 26, wherein the enzyme is a
2 DNA or RNA ligase.

- 1 28. The method of Claim 27, wherein the DNA ligase is
2 selected from T₄ DNA ligase or Taq DNA ligase.
- 1 29. The method of Claim 26, wherein the chemical agent
2 is cyanogen bromide or a carbodiimide.
- 1 30. The method of any of Claims 1-10 further
2 comprising subjecting the paramagnetic particles
3 to a magnetic field sufficient to prevent loss of
4 the particles from the reaction vessel during
5 separating and washing steps, wherein the bound
6 ligated amplification sequence remains in the
7 reaction vessel upon removal of unbound materials.
- 1 31. The method of any of Claims 1-10, wherein the
2 target nucleic acid is selected from the group
3 consisting of HIV-1 RNA, Mycobacteria ribosomal
4 RNA and HCV RNA.
- 1 32. The method of Claim 31, wherein the ribosomal RNA
2 is 16S rRNA from the group consisting of
3 Mycobacteria spp., M. tuberculosis, M. avium and
4 M. intracellulaire.
- 1 33. The method according to any of Claims 2, 4, 6, 8
2 and 10, wherein the step of amplifying the
3 amplification sequence is carried out using an
4 amplification technique selected from the group
5 consisting of polymerase chain reaction (PCR),
6 strand displacement amplification (SDA), ligase
7 chain reaction (LCR), self-sustained sequence
8 reaction (3SR) and Q β replicase amplification
9 (Q β RA).
- 1 34. The method according to Claim 2, wherein the
2 capture/amplification probe comprises at its 3'

3 end a nucleotide sequence recognized by at least
4 one restriction endonuclease.

1 35. The method according to Claim 34 further
2 comprising hybridizing an oligonucleotide
3 releasing primer to the 3' end of the bound
4 ligated amplification sequence, said releasing
5 primer comprising a nucleotide sequence
6 complementary and hybridizable to the nucleotide
7 sequence in the ligated amplification sequence
8 recognized by one or more restriction
9 endonucleases such that a double stranded molecule
10 is formed comprising one or more restriction
11 endonuclease cleavage sites and cleaving the
12 double-stranded molecule with at least one
13 restriction endonuclease, wherein the cleavage
14 thereof releases the ligated amplification
15 sequence from the paramagnetic particles.

1 36. The method according to any of Claims 3-10,
2 wherein more than one different target nucleic
3 acid in a single sample may be detected.

1 37. A method for in situ detection of a target nucleic
2 acid in a histological specimen comprising the
3 steps of:
4 (a) preparing a tissue sample from the
5 histological specimen to be analyzed for the
6 presence of the target nucleic acid;
7 (b) washing said tissue sample;
8 (c) adding one or more amplification probes
9 to said tissue sample, said amplification probes
10 each having a region of its nucleotide sequence
11 that is complementary to the target nucleic acid
12 to be detected and a region that is not
13 complementary to the target nucleic acid sequence,

14 wherein if more than one probe is used, the
15 complementary sequences are hybridizable to
16 adjacent non-overlapping sequences in the
17 nucleotide sequence of the target nucleic acid,
18 and incubating the probes and tissue sample under
19 conditions that allow for hybridization of the
20 probe or probes to the target nucleic acid to
21 occur;
22 (d) washing unbound probe or probes from the
23 tissue sample;
24 (e) amplifying the bound amplification
25 probes under conditions, wherein any unbound
26 probes are not amplified; and
27 (f) detecting the amplified probe sequences,
28 wherein the detection thereof indicates the
29 presence of the target nucleic acid in the tissue
30 sample.

1 38. The method of Claim 37 further comprising ligating
2 the amplification probes prior to amplification.

1 39. The method of Claims 37 or 38, wherein
2 amplification is carried out using a PCR
3 amplification technique.

1 40. The method of Claim 39, wherein a signal
2 generating moiety is attached to nucleotides
3 utilized in the amplification technique.

1 41. The method of Claim 40, wherein the signal
2 generating moiety is digoxinin.

1 42. A kit for use in capturing and detecting the
2 presence of a target nucleic acid in a clinical
3 sample comprising in packaged combination:

4 (a) a capture/amplification probe containing
5 a ligand covalently attached to its 3' end and
6 having a 3' nucleotide sequence that does not
7 hybridize to the nucleotide sequence of the target
8 nucleic acid and a 5' nucleotide sequence
9 complementary and hybridizable to the nucleotide
10 sequence of the target nucleic acid;

11 (b) an amplification probe having a 3'
12 nucleotide sequence complementary to a nucleotide
13 sequence in the target nucleic acid immediately
14 adjacent the sequence complementary to the 5' end
15 of the first probe and a 5' end that does not
16 hybridize to the target nucleic acid;

17 (c) paramagnetic particles coated with a
18 ligand binding moiety that binds to the ligand on
19 the capture/amplification probe.

1 43. A kit for use in capturing and detecting the
2 presence of a target nucleic acid in a clinical
3 sample comprising in packaged combination:

4 (a) one or more capture/amplification
5 probes, each probe having a ligand bound to the 3'
6 or 5' end thereof and having a region of its
7 nucleotide sequence that is complementary to the
8 nucleotide sequence of the target nucleic acid and
9 a region that is non-complementary to the target
10 nucleic acid, wherein the ligand is bound to the
11 non-complementary region of the probe;

12 (b) a pair of amplification probes, each of
13 which having a region of nucleotide sequence that
14 is complementary and hybridizable to adjacent and
15 non-overlapping regions of the target nucleic acid
16 sequence and a region of nucleotide sequence that
17 is non-complementary to the target nucleic acid;

18 (c) paramagnetic particles coated with a ligand
19 binding moiety that binds to the ligand on the
20 capture/amplification probe.

- 1 44. A kit for use in capturing and detecting the
2 presence of a target nucleic acid in a clinical
3 sample comprising in packaged combination:
4 (a) one or more capture/amplification
5 probes, each probe having a ligand bound to the 3'
6 or 5' end thereof and having a region of its
7 nucleotide sequence that is complementary to the
8 nucleotide sequence of the target nucleic acid and
9 a region that is non-complementary to the target
10 nucleic acid, wherein the ligand is bound to the
11 non-complementary region of the probe;
12 (b) an amplification sequence having 3' and
13 5' regions of nucleotide sequence that are non-
14 complementary to the target nucleic acid flanking
15 a region that is complementary and hybridizable to
16 the target nucleic acid;
17 (c) paramagnetic particles coated with a
18 ligand binding moiety that binds to the ligand on
19 the capture/amplification probe.

- 1 45. A kit for use in capturing and detecting the
2 presence of a target nucleic acid in a clinical
3 sample comprising in packaged combination:
4 (a) one or more capture/amplification
5 probes, each probe having a ligand bound to the 3'
6 or 5' end thereof and having a region of its
7 nucleotide sequence that is complementary to the
8 nucleotide sequence of the target nucleic acid and
9 a region that is non-complementary to the target
10 nucleic acid, wherein the ligand is bound to the
11 non-complementary region of the probe;

12 (b) an amplification sequence having 3' and
13 5' regions of nucleotide sequence that are
14 complementary and hybridizable to adjacent
15 sequences in the target nucleic acid, said 3' and
16 5' regions flanking a region of the amplification
17 sequence that is non-complementary to the target
18 nucleic acid;

19 (c) paramagnetic particles coated with a
20 ligand binding moiety that binds to the ligand on
21 the capture/amplification probe.

1 46. The kit of any of Claims 42-45, wherein the ligand
2 is biotin and the ligand binding moiety is
3 streptavidin.

1 47. The kit of any of Claims 42-45, wherein the
2 capture/amplification probe is an oligonucleotide
3 the sequence of which is selected from the group
4 consisting of SEQ ID NO. 1, SEQ ID NO. 7, SEQ ID
5 NO. 10, SEQ ID NO. 13, SEQ ID NO. 16., SEQ ID NO.
6 22 and SEQ ID NO. 23.

1 48. The kit of Claim 42, wherein the amplification
2 probe is an oligonucleotide the sequence of which
3 is selected from the group consisting of SEQ ID
4 NO. 2, SEQ ID NO. 8, SEQ ID NO. 11, SEQ ID NO. 14
5 and SEQ ID NO. 17.

1 49. The kit of Claim 43, wherein the amplification
2 probe is an oligonucleotide the sequence of which
3 is selected from the group consisting of SEQ ID
4 NO. 24 and SEQ ID NO. 25.

1 50. The kit of Claim 44, wherein the amplification
2 sequence is an oligonucleotide sequence consisting
3 of SEQ ID NO. 30.

- 1 51. The kit of Claim 45, wherein the amplification
- 2 sequence is an oligonucleotide sequence consisting
- 3 of SEQ ID NO. 31.

1/6

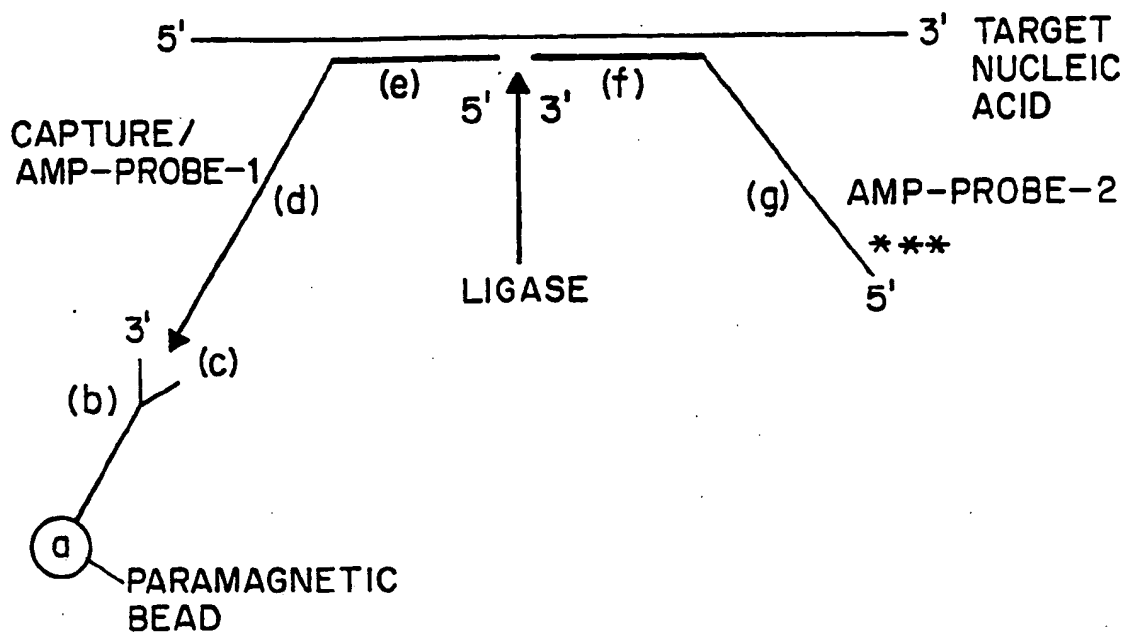


FIG. 1

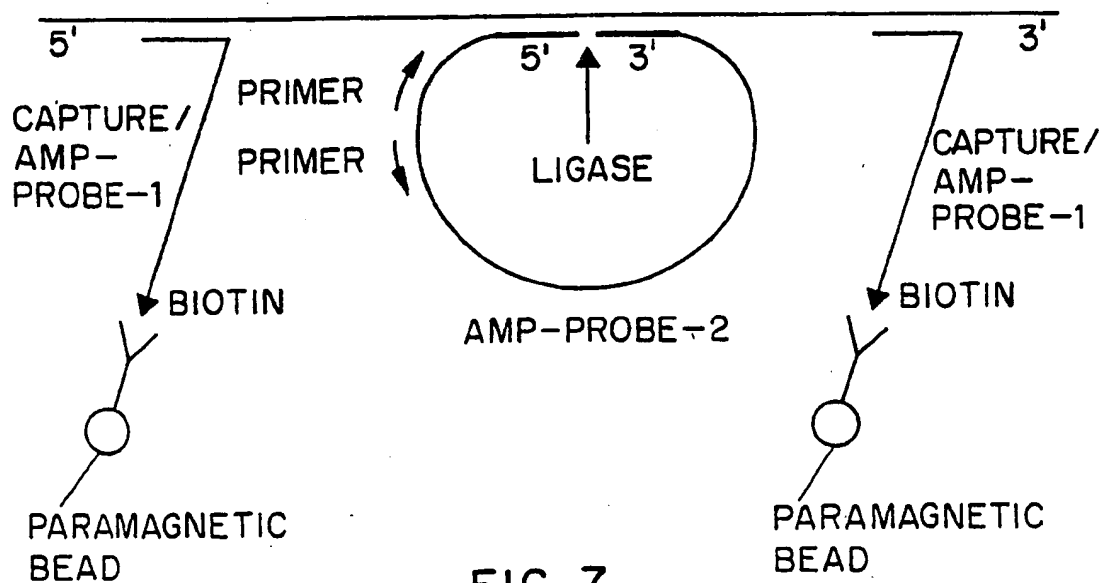


FIG. 7

SUBSTITUTE SHEET (RULE 26)

2/6

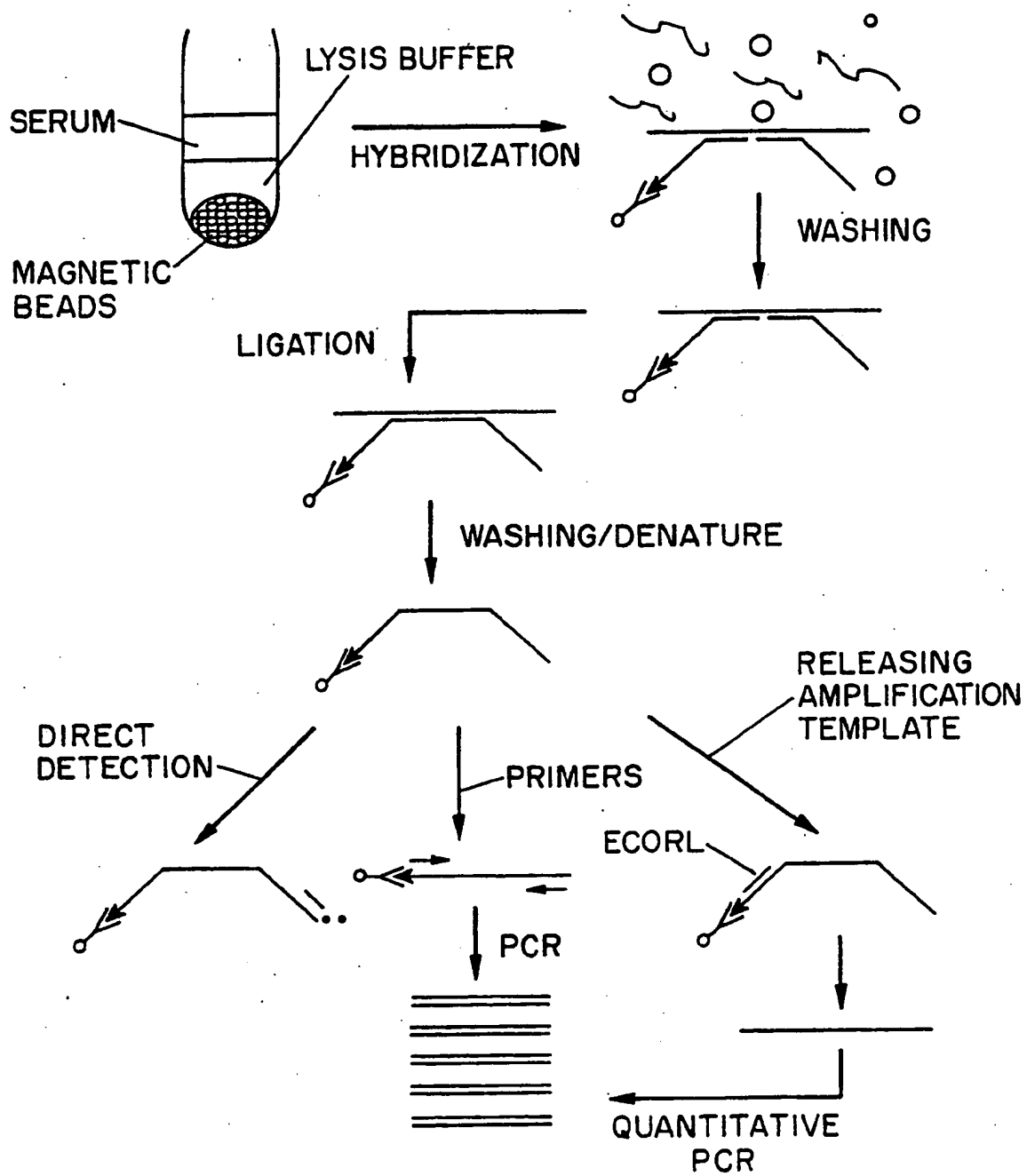


FIG. 2

3/6

A B

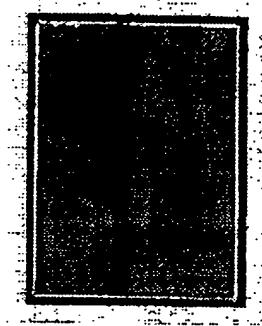


FIG. 3

4/6

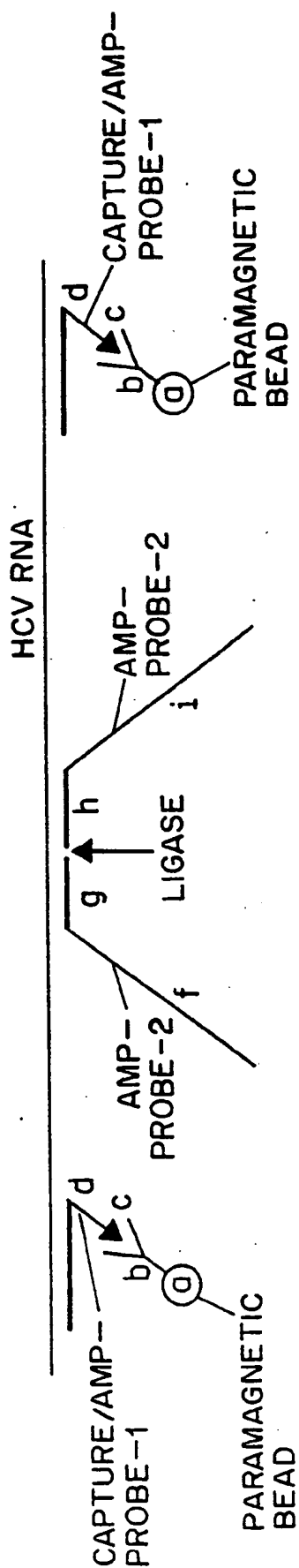


FIG. 4

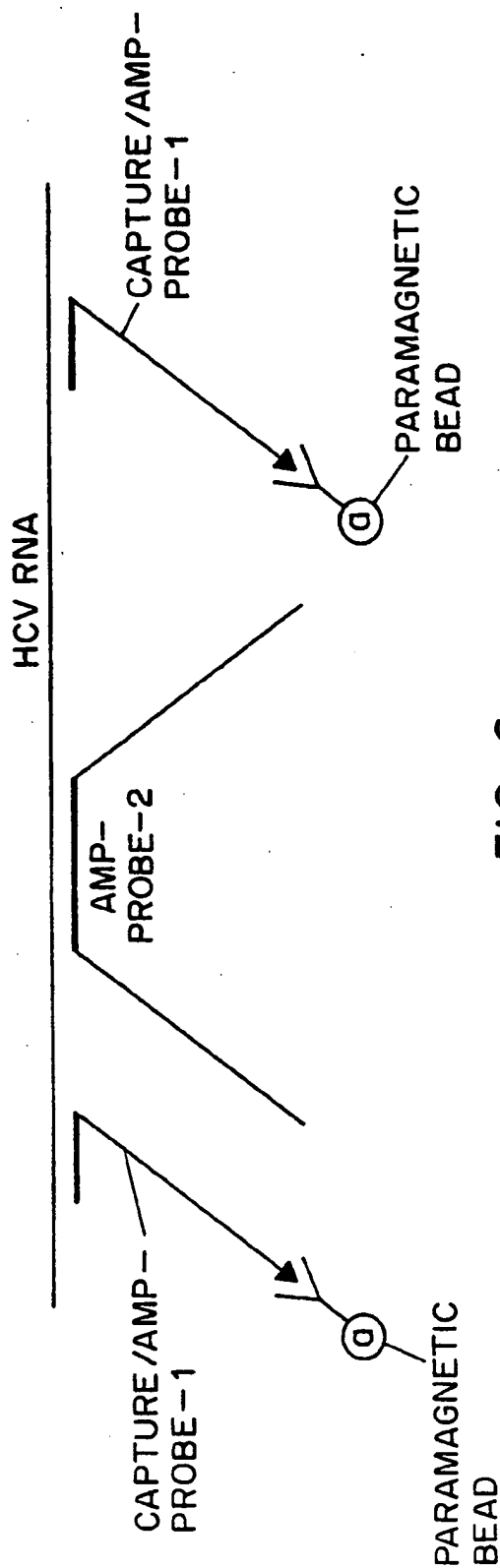


FIG. 6

5/6

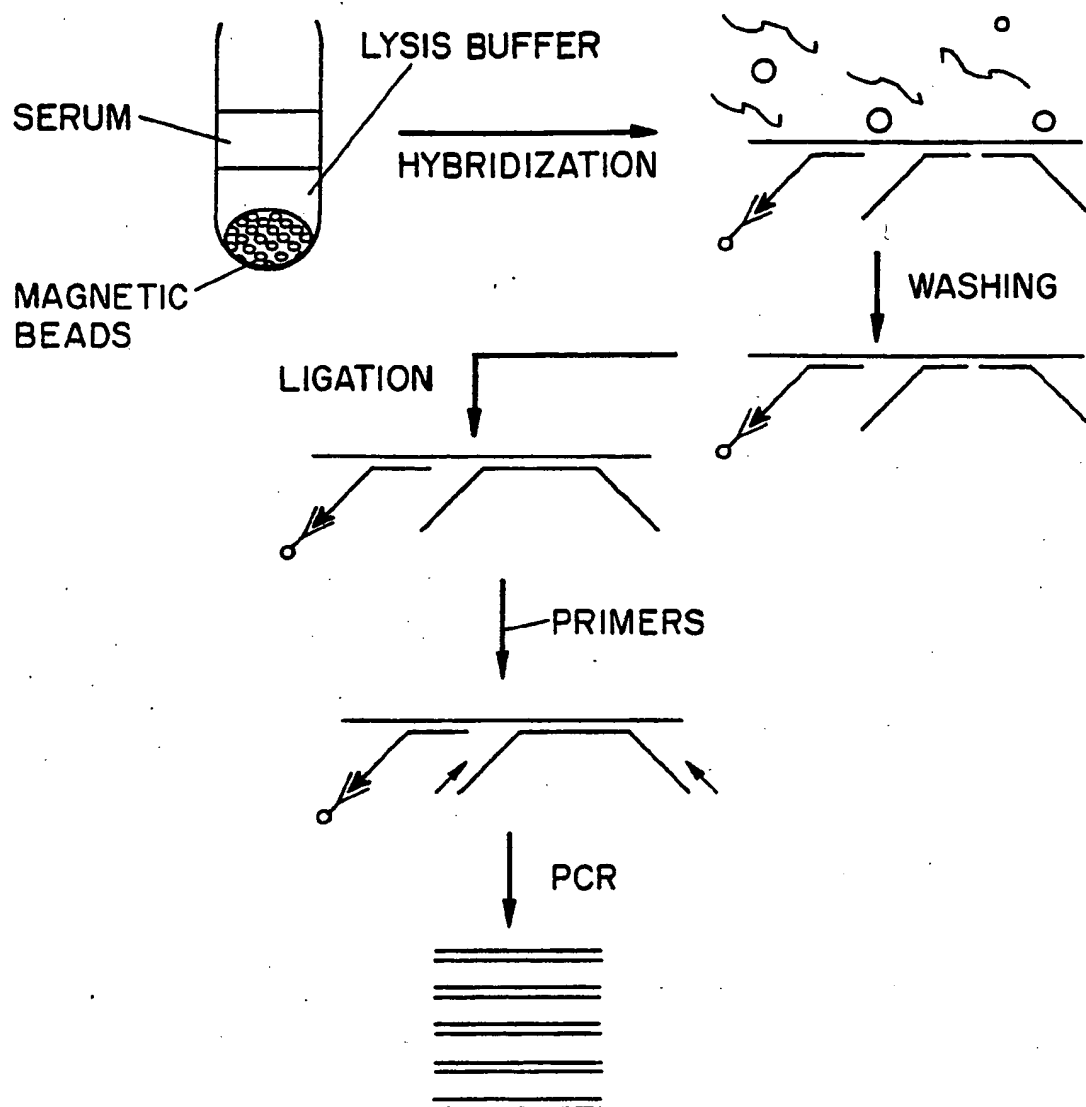


FIG. 5

6/6

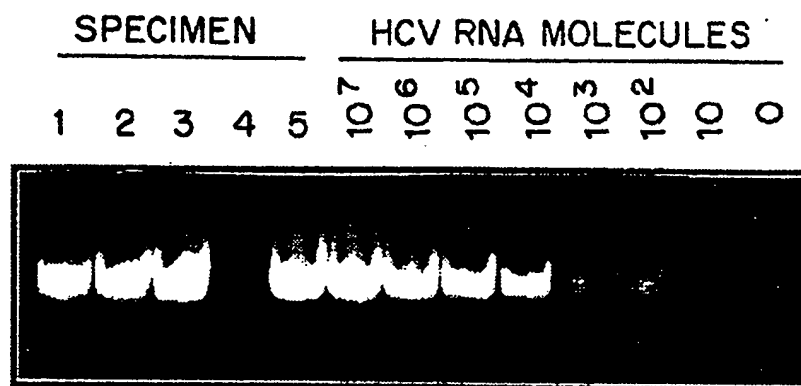


FIG. 8

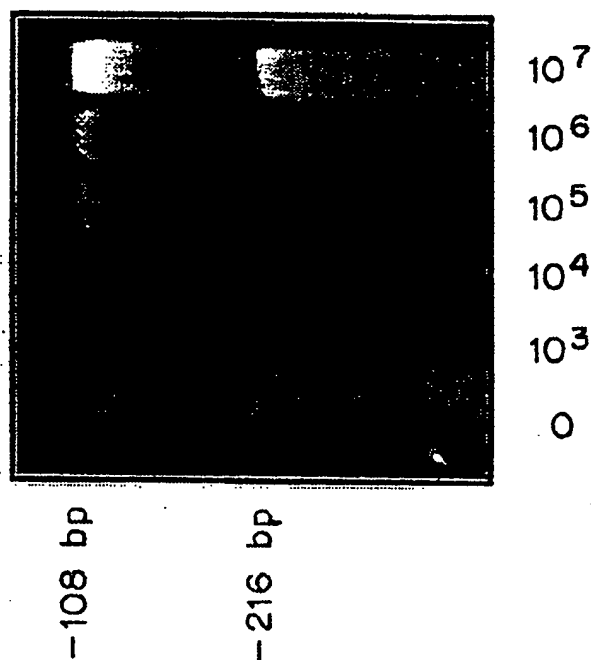


FIG. 9

SUBSTITUTE SHEET (RULE 26)

BEST AVAILABLE COPY

INTERNATIONAL SEARCH REPORT

Internat. Application No.

PCT/US 95/07671

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/68 C07H21/04

C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 324 616 (AMACO CORP.) 19 July 1989 see page 7, line 58 - page 9, line 14 ---	1-46
Y	EP,A,0 357 336 (SYNTEX (USA) INC.) 7 March 1990 see page 13, line 38 - page 15, line 48 ---	1-46
Y	EP,A,0 185 494 (APPLIED BIOSYSTEMS INC.) 25 June 1986 see page 9 - page 10 ---	1-46
Y	EP,A,0 481 704 (CIBA CORNING DIAGNOSTICS INC) 22 April 1992 see figures 2,3 ---	1-46
	--- -/-	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

6 October 1995

Date of mailing of the international search report

09.11.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2220 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+ 31-70) 340-3016

Authorized officer

Osborne, H

INTERNATIONAL SEARCH REPORT

Internat. Application No.

PCT/US 95/07671

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOTECHNIQUES, vol. 16, no. 2, February 1994 pages 238,240--241, AKANE A ET AL 'direct dideoxy sequencing of genomic DNA by ligation mediated PCR' see figure 1 ----	1-46
Y	WO,A,90 01065 (GENELABS INC) 8 February 1990 see figures 1-6 ----	1-46
A	PROC NATL ACAD SCI, vol. 87, no. 22, November 1990 pages 8923-27, NICKERSON D ET AL 'automated DNA diagnostics using an elisa based ligation assay' see the whole document -----	1-46
A	WO,A,93 13223 (CHIRON CORP) 8 July 1993 -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat'l Application No

PCT/US 95/07671

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-324616	19-07-89	US-A- 5219734	15-06-93
		AT-T- 120496	15-04-95
		AU-B- 2848589	27-07-89
		DE-D- 68921901	04-05-95
		DE-T- 68921901	24-08-95
		JP-A- 2005898	10-01-90
		US-A- 5449602	12-09-95
EP-A-357336	07-03-90	US-A- 5185243	09-02-93
		DE-D- 68918657	10-11-94
		DE-T- 68918657	18-05-95
		JP-A- 2135100	23-05-90
EP-A-185494	25-06-86	US-A- 4883750	28-11-89
		DE-A- 3586090	25-06-92
		JP-B- 6044880	15-06-94
		JP-A- 61191964	26-08-86
		US-A- 5242794	07-09-93
EP-A-481704	22-04-92	AU-B- 635142	11-03-93
		AU-B- 8045391	30-04-92
		JP-A- 5068597	23-03-93
		US-A- 5407798	18-04-95
WO-A-9001065	08-02-90	AU-B- 632760	14-01-93
		AU-B- 4049589	19-02-90
		EP-A- 0426756	15-05-91
		JP-T- 4501353	12-03-92
WO-A-9313223	08-07-93	JP-T- 7502654	23-03-95